ABERRANT EPIGENETICS IN THE MOLECULAR PATHOGENESIS OF HUMAN ACUTE MYELOID LEUKEMIA

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Saskatoon

by

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ABSTRACT

Promoter hypermethylation mediated gene silencing is a frequent epigenetic finding in many cancers that affects genes known to have important roles in several aspects of cell biology. Hematological malignancies have been reported to harbor multiple genes aberrantly silenced by promoter hypermethylation and as a result, cytosine analogs known to inhibit the DNA methylation machinery are currently being evaluated in clinical trials. As such, the general goal of this thesis was to identify genes silenced by promoter hypermethylation in human acute myeloid leukemia (AML) and to study the mechanism of promoter hypermethylation mediated gene silencing. Interestingly, the cyclin dependent kinase inhibitor p15 was found to be methylated at a high frequency in AML patients and cell lines in association with a lack of detectable p15 mRNA. Treatment with the cytosine analog 5-Aza-2'-deoxycytidine (5-Aza-dC) *in vitro* resulted in promoter demethylation and p15 mRNA re-expression, which was associated with a release of a transcriptionally repressive complex at the p15 promoter. Importantly, 5-Aza-dC treatment also reversed specific histone amino-terminal modifications at the p15 promoter which are normally associated with transcriptionally inactive chromatin regions, implicating chromatin remodeling in promoter hypermethylation mediated gene silencing. The recently discovered DNA methylation inhibitor, zebularine – considered more stable than 5-Aza-dC – was also able to reconstitute p15 mRNA *in vitro* in association with promoter demethylation, regional enrichment of histone acetylation, and growth inhibition.

To identify novel genes silenced by promoter hypermethylation in AML, cDNA microarray analysis was employed following *in vitro* pharmacological inhibition of DNA methylation and histone deacetylation. Of note, four genes from the metallothionein family of cysteine rich small molecules were consistently upregulated following drug treatment and further evaluation identified the gene MT1H to be hypermethylated at a high frequency in AML patients and cell lines. Taken together, the data suggests that aberrant promoter hypermethylation mediated gene silencing occurs in multiple genes from different gene families during the molecular pathogenesis of human AML. Furthermore, the mechanism of promoter methylation mediated transcriptional silencing acts in concert with specific histone modifications which, importantly, can be reversed by treatment with pharmacological inhibitors of DNA methylation.

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TABLE OF CONTENTS

PERMISSION TO USE i

ABSTRACT ii

ACKNOWLEDGEMENTS iii

TABLE OF CONTENTS iv

LIST OF TABLES viii

LIST OF FIGURES ix

LIST OF ABBREVIATIONS xi

1. REVIEW OF THE LITERATURE 1

1.1. INTRODUCTION 1

1.2. ACUTE MYELOID LEUKEMIA 3
- 1.2.1. Epidemiology and Etiology 3
- 1.2.2. Acute Leukemia Classification 4
- 1.2.3. Pathology 7
- 1.2.4. The Molecular Basis of AML 7
  - 1.2.4.1. Impairment of Differentiation 7
  - 1.2.4.2. Activation of Proliferation 11

1.3. THE MOLECULAR BASIS OF CANCER 14
- 1.3.1. Oncogenes 14
- 1.3.2. Tumor Suppressor Genes 16
- 1.3.3. Apoptosis Regulation 18
- 1.3.4. DNA Repair Regulation 19
- 1.3.5. Cell Cycle Regulation 20
- 1.3.6. Cyclin Dependent Kinase Inhibitors 23
  - 1.3.6.1. The CIP/KIP CDKI Family 24
  - 1.3.6.2. The INK4 CDKI Family 26
- 1.3.7. Multistep Molecular Carcinogenesis 30

1.4. EPIGENETICS 32
- 1.4.1. DNA Methylation 32
  - 1.4.1.1. Global Hypomethylation 35
  - 1.4.1.2. CpG Island Hypermethylation 37
  - 1.4.1.3. Genomic Imprinting in Human Cancer 39
  - 1.4.1.4. Mammalian DNA Methyltransferases 41
  - 1.4.1.5. Mammalian Methyl-CpG Binding Domain Proteins 44
- 1.4.2. Post-translational Histone Modification 47
  - 1.4.2.1. Nucleosomes and Chromatin Organization 48

1.4.2.2. Histone Acetylation 50
1.4.2.3. Histone Phosphorylation 54
1.4.2.4. Histone Methylation 55
1.4.2.5. Histone Ubiquitination and ADP-ribosylation 58
1.4.3. DNA Hypermethylation and Chromatin Remodeling 59
1.4.4. Inhibitors of DNA Methylation and Histone Deacetylation 62
1.4.4.1. DNA Methyltransferase Inhibition 62
1.4.4.2. Histone Deacetylase Inhibition 66

2. HYPOTHESIS AND SPECIFIC AIMS 71

3. MATERIALS AND METHODS 74

3.1. Reagents and Suppliers 74

3.2. Oligonucleotides 78

3.3. Antibodies 81

3.4. Patient Specimens 81

3.5. Cell Lines and Tissue Culture 82
3.5.1. Cell Lines and Standard Culture Conditions 82
3.5.2. DNA Methylation Inhibitor Treatments 83
3.5.3. Histone Deacetylase Inhibitor Treatments 84

3.6. General Molecular Techniques 84
3.6.1. Agarose Gel Electrophoresis 84
3.6.1.1. Standard Agarose Gel Electrophoresis 84
3.6.1.2. Formaldehyde Agarose Gel Electrophoresis 85
3.6.2. Generation of 3' End Labeled Oligonucleotide Probes 85
3.6.3. Small Scale Isolation of Plasmid DNA from Bacterial Cells 86
3.6.4. Isolation of Total RNA from Eukaryotic Cells 86
3.6.5. Isolation of DNA from Eukaryotic Cells 86
3.6.6. Isolation of Protein Lysates from Eukaryotic Cells 87
3.6.7. Isolation of Histone Proteins from Cultured Eukaryotic Cells 87

3.7. Reverse Transcriptase PCR (RTPCR) 88

3.8. RNase Protection Assay (RPA) 89

3.9. DNA Methylation Analysis 90
3.9.1. Sodium Bisulfite Treatment of Genomic DNA 90
3.9.2. Methylation Specific PCR (MSP) 90
3.9.3. Temporal Temperature Gradient Gel Electrophoresis (TTGE) 91

3.10. DNA Sequencing 93
3.10.1. Direct PCR Product Sequencing 93
3.10.2. TTGE Band Recovery and Sequencing 93

3.11. Chromatin Immunoprecipitation (ChIP) 94

3.12. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) 96

3.13. Western Blotting 97

3.14. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] Assay 98

3.15. Tritiated Thymidine Incorporation Assay 98

3.16. Propidium Iodide (PI) Staining and Flow Cytometry 99

3.17. cDNA Microarray Analysis 99
3.17.1. Sample Preparation 100
3.17.2. Direct Labeling with Cyanine 3- and Cyanine 5-dCTP 100
3.17.3. Probe Hybridization 101
3.17.4. Microarray Washing 101
3.17.5. Microarray Scanning 102
3.17.6. Data Analysis 102

4. RESULTS AND DISCUSSION 103

4.1. METHYLATION STATUS OF CDKI GENES IN HUMAN AML 103

4.1.1. CDKI Expression in Human AML Cell Lines and Patient Samples 103
4.1.2. p15 Promoter Hypermethylation in Human AML 105
4.1.3. p21 Promoter Hypermethylation in Human AML 110
4.1.4. p27 Promoter Hypermethylation in Human AML 112
4.1.5. DISCUSSION 113

4.2. DEMETHYLATION AND RE-EXPRESSION OF p15 BY 5-Aza-dC IS ASSOCIATED WITH H3 MODIFICATIONS AND RELEASE OF MeCP2 AND SUV39H1 AT THE PROMOTER 121

4.2.1. 5-Aza-dC Demethylates and Induces p15 in AML193 Cells 121
4.2.2. 5-Aza-dC Mediated Induction of p15 is Associated with MeCP2 and SUV39H1 Release at the Promoter 122
4.2.3. 5-Aza-dC Mediated Induction of p15 is Associated with Changes 126

in H3 Acetylation
4.2.4. 5-Aza-dC Mediated Induction of p15 is Associated with Changes in H3 Methylation 127
4.2.5. 5-Aza-dC Mediated Changes in H3 Modifications are Gene Specific 130
4.2.6. 5-Aza-dC and TSA Combinatorial Treatment Synergistically Induces p15 in AML193 Cells 132
4.2.7. DISCUSSION 136

4.3. ZEBULARINE INHIBITS HUMAN AML GROWTH AND p15 PROMOTER HYPERMETHYLATION 143

4.3.1. Zebularine Induces and Demethylates p15 in AML193 Cells 143
4.3.2. Zebularine Mediated Induction of p15 is Associated with Regional Histone Acetylation Enrichment 146
4.3.3. Zebularine is Cytotoxic and Inhibits AML193 Proliferation 148
4.3.4. Zebularine Induces Apoptosis and Halts AML193 Cells at G2/M 150
4.3.5. Zebularine Induces Global Histone Phosphorylation in AML193 Cells 152
4.3.6. DISCUSSION 155

4.4. cDNA MICROARRAY ANALYSIS OF GENES UPREGULATED BY TREATMENT WITH 5-Aza-dC IN COMBINATION WITH TSA IDENTIFIES ABERRANT MT1H PROMOTER HYPERMETHYLATION AT A HIGH FREQUENCY IN HUMAN AML 160

4.4.1. cDNA Microarray Analysis of AML193 Cells Following DNMT and HDAC Inhibition 160
4.4.2. Methylation Analysis of Candidate Genes in AML193 Cells 164
4.4.3. Methylation Analysis of Metallothionein Genes in AML 166
4.4.4. 5-Aza-dC Mediated Induction of MT1H is Associated with Changes in H3 Methylation and Acetylation 168
4.4.5. DISCUSSION 170

5. CONCLUSIONS AND FUTURE STUDIES 178

6. REFERENCES 185

LIST OF TABLES

Table 1.1 FAB Classification of AML and Incidence 6

Table 3.1 Reagents and Suppliers Used in This Study 74

Table 3.2 Commercially Available Kits Used in This Study 76

Table 3.3 Names and Addresses of Suppliers 77

Table 3.4 Sequences and Optimal Annealing/Hybridization Temperatures of Primers and Probes Used in This Study 78

Table 3.5 Antibodies Used in This Study 81

Table 3.6 Cell Lines Used in This Study 83

Table 4.1 Genes Upregulated by 5-Aza-dC and TSA Treatment in AML193 Cells 162

LIST OF FIGURES

Figure 1.1 Mammalian Hematopoiesis 9

Figure 1.2 Regulation of the Eukaryotic Cell Cycle 21

Figure 1.3 CIP/KIP CDKI Proteins 25

Figure 1.4 INK4 CDKI Proteins 27

Figure 1.5 INK4 Genomic Locus at 9p21 29

Figure 1.6 Pyrimidine Nucleoside and 5-Methyl-cytidine Structures 33

Figure 1.7 Known Post-Translational Histone Modifications 51

Figure 1.8 Model of DNA Hypermethylation Mediated Gene Silencing 60

Figure 1.9 Chemical Structures of Common DNMT Inhibitors 63

Figure 1.10 Chemical Structures of Common HDAC Inhibitors 67

Figure 4.1 CDKI Expression in Human AML 104

Figure 4.2 p15, p21, and p27 MSP, TTGE, and ChIP PCR Product Locations 107

Figure 4.3 p15 Methylation in Human AML 108

Figure 4.4 p21 Methylation in Human AML 111

Figure 4.5 p27 Methylation in Human AML 114

Figure 4.6 Demethylation and Re-expression of p15 by 5-Aza-dC Treatment in AML193 Cells 123

Figure 4.7 Epigenetic Proteins at the p15 Promoter in AML193 Cells 125

Figure 4.8 H3 Acetylation at the p15 Promoter in AML193 Cells 128

Figure 4.9 H3 Methylation at the p15 Promoter in AML193 Cells 131

Figure 4.10 Effects of 5-Aza-dC on Global H3 Modifications in AML193 133

Cells

Figure 4.11 Effects of 5-Aza-dC and TSA Combinatorial Treatment in AML193 Cells 135

Figure 4.12 Summary of ChIP Data Before and After 5-Aza-dC Treatment 137

Figure 4.13 Zebularine Treatment in AML193 Cells 145

Figure 4.14 ChIP Analysis of H3 and H4 Acetylation Following Zebularine Treatment in AML193 Cells 147

Figure 4.15 MTT and Tritiated Thymidine Assays Following Zebularine Treatment in AML193 Cells 149

Figure 4.16 Effects of Zebularine on the Cell Cycle in AML193 Cells 151

Figure 4.17 Zebularine Induces Global Histone Phosphorylation in AML193 Cells 154

Figure 4.18 Semi-quantitative RTPCR of Candidate Genes Identified by cDNA Microarrays 163

Figure 4.19 MSP of Candidate Genes Following Treatment with 5-Aza-dC 165

Figure 4.20 16q13 Locus and Metallothionein Amino Acid Alignment 167

Figure 4.21 Metallothionein Promoter Hypermethylation 169

Figure 4.22 MT1H ChIP Analysis of H3 Lysine 9 Methylation and Acetylation 171

LIST OF ABBREVIATIONS

5-Aza	5-Azacytidine
5-Aza-dC	5-Aza-2'-deoxycytidine
ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
APC	adenomatous polyposis coli
APL	acute promyelocytic leukemia
ARF	alternative reading frame
APS	ammonium persulfate
ATRA	all-*trans*-retinoic acid
BSA	bovine serum albumin
BWS	Beckwith-Wiedemann syndrome
CBF	core binding factor
CDK	cyclin-dependent kinase
CDKI	cyclin-dependent kinase inhibitor
C/EBPα	CCAAT/enhancer binding protein-alpha
ChIP	chromatin immunoprecipitation
CML	chronic myeloid leukemia
DEPC	diethyl pyrocarbonate
DGGE	denaturing gradient gel electrophoresis
DNMT	DNA methyltransferase
dATP	deoxyadenosine 5'-triphosphate
dCTP	deoxycytidine 5'-triphosphate

dGTP	deoxyguanosine 5’-triphosphate
dNTP	deoxynucleotide triphosphate
dTTP	thymidine 5’-triphosphate
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
EMBOSS	European Molecular Biology Open Software Suite
EST	expressed sequence tag
FAB	French-American-British
FBS	fetal bovine serum
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GPX4	glutathione peroxidase 4
H1	histone H1
H2A	histone H2A
H2A.X	histone variant H2A.X
H2B	histone H2B
H3	histone H3
H4	histone H4
HAT	histone acetyltransferase
HDAC	histone deacetylase
HEPES	hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HMT	histone methyltransferase
HNPCC	hereditary nonpolyposis colon cancer
HNSCC	head and neck squamous cell carcinoma

HP1	heterochromatic protein 1
HRP	horseradish peroxidase
IGI30	interferon-gamma inducible protein 30
IGF2	insulin-like growth factor 2
ITD	internal tandem duplication
LB	Luria-Bertani
LOH	loss of heterozygosity
LOI	loss of imprinting
LOWESS	locally weighted regression scatter plot smoothing
MBD	methyl-CpG binding domain
MDR1	multidrug-resistance gene 1
MDS	myelodysplastic syndrome
M-MLV-RT	Moloney murine leukemia virus-reverse transcriptase
MMR	mismatch repair
MOPS	3-(N-morpholino) propane sulfonic acid
MSI	microsatellite instability
MSP	methylation specific PCR
MT1B	metallothionein 1B
MT1E	metallothionein 1E
MT1G	metallothionein 1G
MT1H	metallothionein 1H
MT1L	metallothionein 1L
MT2A	metallothionein 2A

MT3	metallothionein 3
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NuRD	nucleosome remodeling and histone deacetylation
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PI	propidium iodide
PMA	phorbol 12-myristate 13-acetate
PMSF	phenylmethylsulfonyl fluoride
RARα	retinoic acid receptor-alpha
RB	retinoblastoma
RIPA	radioimmunoprecipitation
RPA	RNase protection assay
RTPCR	reverse transcription-polymerase chain reaction
SAHA	suberoylanilide hydroxamic acid
SAT	spermidine/spermine N1-acetyltransferase
SDS	sodium dodecyl sulfate
SSC	sodium chloride/sodium citrate
STAT	signal transducer and activator of transcription
TAE	Tris acetate EDTA
TBE	Tris borate EDTA
TBS	Tris buffered saline

TdT	terminal deoxynucleotidyl transferase
TE	Tris EDTA
TEMED	N,N,N,N-tetramethyl-ethylenediamine
TGF-β1	transforming growth factor β-1
TGFβRII	TGF-β receptor type-II
TSA	trichostatin A
TTGE	temporal temperature gradient gel electrophoresis
UTP	uridine 5'-triphosphate
WBC	white blood cell
WHO	World Health Organization

1. REVIEW OF THE LITERATURE

1.1. INTRODUCTION

Cancer has been historically considered a genetic disease and human leukemia, which is primarily associated with hallmark chromosomal translocations, is no exception. However, although much is known about many of the underlying genetic abnormalities in cancer, aside from the discovery of imatinib mesylate for the treatment of chronic myeloid leukemia (CML), little progress has been made in exploiting cancer genetics for clinical gain.

The field of molecular biology known as 'epigenetics' involves the study of heritable changes in gene expression that occur without any alteration in DNA sequence and, importantly, deregulation of this process has been implicated in many diseases and inherited syndromes, including cancer. The recent expansion of knowledge regarding cancer epigenetics has influenced the cancer research community twofold: 1) it has modified the previous cancer paradigm, whereby cancer is now viewed as a genetic *and* epigenetic disease (Feinberg, 2001), and 2) it has exposed a new direction for experimental therapy (Egger et al., 2004).

The general goal of this thesis is to investigate the presence of aberrant epigenetics in human acute myeloid leukemia (AML) and to study the mechanisms that are involved in epigenetic gene silencing. As such, the following review of the

literature aims to familiarize the reader with AML, the molecular basis of human cancer, and the general concepts of epigenetics. Emphasis will be placed on cell cycle regulation and its role in cancer, DNA methylation mediated transcriptional silencing, and the exciting prospect of pharmacologic inhibition of aberrant epigenetic gene silencing.

1.2. ACUTE MYELOID LEUKEMIA

1.2.1. Epidemiology and Etiology

AML is a malignant neoplasm of hematopoietic stem cells originating in and infiltrating the bone marrow. The clonal accumulation of leukocyte precursors known as leukemic blasts that are characteristic of AML often results in pancytopenia. In most cases the disease involves the peripheral blood but it can involve any organ including the spleen, liver, and lymph nodes. The disease primarily occurs in adults, shows an equal frequency in males and females, and like many cancers, the incidence rises steeply with increasing age (Alderson, 1980; Clark and Macmahon, 1956). The median age of AML diagnosis is 64 years and it is the most common adult leukemia, accounting for roughly half of all leukemias (Alderson, 1980; Appelbaum et al., 2001).

The etiology of leukemia is unknown; however, environmental factors such as ionizing radiation and chemical exposure have been associated with the disease. Hereditary factors are implicated in leukemia etiology as evidenced by a tendency for the disease to cluster within some families and an association with constitutional chromosomal abnormalities (Zuelzer and Cox, 1969). Furthermore, clinical observations have identified unusual AML susceptibility in monozygotic twins and in children with certain genetic diseases and congenital disorders (Fraumeni and Miller, 1967; Jackson et al., 1969), most notably Down syndrome (Rosner and Lee, 1972). However, like other leukemias and solid tumor malignancies, the etiology of AML likely involves an intricate combination of

hereditary and genetic factors as well as environmental exposures. Given that the precise mechanism by which these potential etiologic factors produce leukemia are as yet unknown, the bulk of knowledge regarding AML etiology remains indirect. To this end, a great deal of effort and research is continually expended with the goal of identifying causative factors of leukemia.

1.2.2. Acute Leukemia Classification

The diagnosis of AML entails a stepwise approach that requires differentiating it from reactive disorders and other neoplastic diseases, such as acute lymphoblastic leukemia (ALL), and then classifying the disease into subcategories that define treatment and prognostic groups. Morphologic assessment of blood and bone marrow smears and marrow biopsy sections accompanied by cytochemical stains such as myeloperoxidase and non-specific esterase can often accurately discriminate between AML and ALL (McKenna, 2000). However, leukemias that are poorly differentiated or whose lineage cannot be discerned using standard morphologic and cytochemical techniques require immunophenotyping to assist in their diagnosis and accurate classification (Griffin et al., 1983). To this end, the lineage of hematopoietic cells is defined both by antigen expression and the absence of antigen expression associated with a unique lineage. In AML, immunophenotyping is most important in distinguishing poorly differentiated cases from ALL and in characterizing AML subsets (McKenna, 2000).

Clonal cytogenetic abnormalities are identified in 60 - 80% of AMLs (Mrozek et al., 1997), and can frequently be of clinical value. This is particularly true of cases with the t(15;17) translocation that is consistently associated with acute promyelocytic leukemia (APL) and often has distinct clinical and morphologic features (Bitter et al., 1987). The reciprocal translocation involves the PML gene on chromosome 15 and the retinoic acid receptor-alpha (RARα) gene on chromosome 17 (Grimwade, 1999), and the resulting fusion mRNA product inhibits maturation of the affected cells leading to a proliferation of atypical promyelocytes. In most cases of APL, treatment with all-*trans*-retinoic acid (ATRA) overcomes the maturation block and induces a temporary complete remission of the disease (Degos et al., 1995). However, standard chemotherapy administered after or with ATRA is often required to sustain remission.

Although immunophenotyping, cytogenetics, and molecular analysis are important in the diagnosis of acute leukemia, the widely used classification scheme proposed by the French-American-British (FAB) Cooperative Group primarily utilizes morphologic assessment to classify acute leukemias (Bennett et al., 1976; Bennett et al., 1985a; Bennett et al., 1985b; Bennett et al., 1991). The FAB classification of AML is a lineage-based morphologic classification that categorizes cases according to the degree of maturation of the leukemic cells and their lineage differentiation (Table 1.1). Although the system is widely accepted internationally, an exclusively cytogenetic/molecular analysis-based classification of acute leukemias, believed to better define biologic and prognostic groupings, is currently being evaluated (Harris et al., 2000; Vardiman et al., 2002).

Table 1.1 FAB Classification and Incidence of AML

AML	Classification	Incidence[a]
Myeloblastic leukemia minimally differentiated	**M0**	<10%
Myeloblastic leukemia without maturation	**M1**	20%
Myeloblastic leukemia with maturation	**M2**	30%
Hypergranular promyelocytic leukemia	**M3**	10%
Myelomonocytic leukemia	**M4**	25%
Myelomonocytic leukemia with eosinophilia	**M4Eo**	<5%
Monocytic leukemia – poorly differentiated	**M5A**	<10%
Monocytic leukemia – differentiated	**M5B**	≤5%
Erythroleukemia	**M6**	≤5%
Megakaryoblastic leukemia	**M7**	≤5%

[a] Based on Tuzuner and Bennett, 1995.

1.2.3. Pathology

As mentioned, AML is diagnosed primarily in adults, although it can occur at any age. An elevated white blood cell (WBC) count is found in approximately one-third of patients with AML at diagnosis, and an equal number of patients have a normal WBC count or leukopenia. Blasts are only present in the peripheral blood of 85% of patients with AML. Thrombocytopenia and a reduced absolute neutrophil count are observed in almost all AML patients at diagnosis. The majority of patients present with a hypercellular bone marrow due to the proliferation of malignant blasts or promyelocytes. According to the original FAB classification scheme, a diagnosis of acute leukemia is made when immature blasts count for at least 30% of all nucleated marrow elements. The more recent World Health Organization (WHO) Classification system requires that blasts account for greater than 20% of all nucleated marrow elements to render a diagnosis of acute leukemia (Jaffe et al., 2001). A marrow containing increased blasts, but accounting for less than 30% of all nucleated marrow elements (or less than 20% in the WHO Classification), usually indicates the presence of a myelodysplastic syndrome (MDS), a diverse group of related disorders characterized by clonal stem cell proliferation, ineffective hematopoiesis, and increased risk to evolve into AML.

1.2.4. The Molecular Basis of AML

1.2.4.1. Impairment of Differentiation

Cloning of translocation breakpoints associated with human leukemias has provided important insights into disease pathogenesis as well as targeted

therapeutics. For example, CML is caused by constitutively activated tyrosine kinases, such as BCR/ABL, that confer a proliferative and survival advantage to hematopoietic progenitors with minimal effects on differentiation. In contrast, the chromosomal translocations commonly associated with AML frequently result in loss-of-function of transcription factors that are required for differentiation and normal hematopoietic development (Tenen, 2003). Hematopoietic development, or hematopoiesis, takes place in the bone marrow and refers to the production of all functioning blood cell types from a pool of pluripotent self-renewing stem cells (Figure 1.1). Some examples of transcription factors involved in hematopoiesis that are often translocated in AML include core binding factor (CBF), the aforementioned RARα, and members of the HOX family of transcription factors (Kelly and Gilliland, 2002).

CBF is a heterodimeric transcription factor composed of a DNA binding component AML1, and CBFβ, a subunit that increases AML1 transcriptional activity without contacting DNA (Coustry et al., 1995). The CBF heterodimer induces expression of a broad spectrum of genes that are critical for hematopoietic development (Speck et al., 1999), as mice deficient in either AML1 or CBFβ are incapable of normal hematopoiesis (Castilla et al., 1996; Okuda et al., 1996). Common translocations that involve CBF are the t(8;21), inv(16), and t(12;21) that result in aberrant expression of the AML1/ETO (Erickson et al., 1992), CBFβ/SMMHC (Liu et al., 1993), and TEL/AML1 (Golub et al., 1995) fusion proteins, respectively. In most cases, expression of the fusion gene dominantly interferes with the function of the residual normal allele, resulting in complete CBF

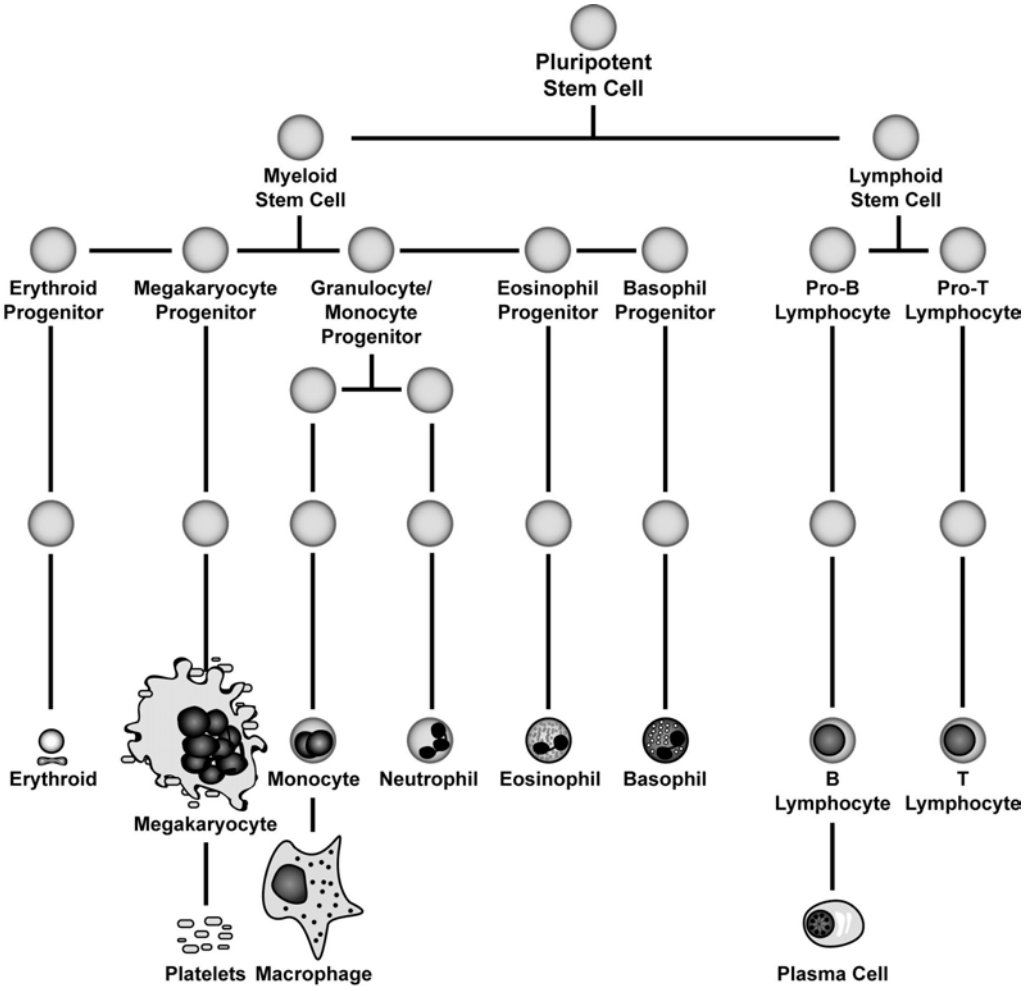

Figure 1.1 Mammalian Hematopoiesis. Illustrated are the various differentiation pathways of the major types of blood cells. Normal hematopoiesis involves the formation of all blood cells originating from a common pluripotent stem cell.

loss-of-function. Interestingly, transcriptional repression of CBF target genes by CBF-related fusion proteins, as well as the aforementioned t(15;17) PML/RARα fusion protein, is frequently mediated in part through abnormal recruitment of a nuclear corepressor complex harboring histone deacetylase (HDAC) activity (Gelmetti et al., 1998; Grignani et al., 1998; Wang and Hiebert, 2001). The concept of histone modification and subsequent chromatin remodeling mediated transcriptional repression is of key interest to the work defined within this thesis and will be expanded upon in subsequent sections.

Although not nearly as prevalent as chromosomal translocations, loss-of-function point mutations in hematopoietic transcription factors also play a role in AML pathogenesis. For example, evidence for mutation of AML1 resulting in both dominant negative activity (Michaud et al., 2002) and haploinsufficiency (Song et al., 1999) exists in association with familial platelet disorder, a disease similar to MDS that often develops into AML. In contrast, mutations in CBFβ have not been found in patients with AML (Leroy et al., 2002), although, as mentioned, the gene is disrupted by inv(16) in some leukemias (Liu et al., 1993). Other loss-of-function mutations associated with AML include the hematopoietic transcription factor CCAAT/enhancer binding protein-alpha (C/EBPα) (Pabst et al., 2001). In some AML patients, point mutations result in a dominant negative C/EBPα allele that is believed to impair normal hematopoiesis, specifically granulocyte differentiation.

Although the hypothesis that inactivating gene rearrangements and transcription factor mutations are causative for AML is attractive, murine models with similar genotypes fail to fully develop comparable leukemic phenotypes

(Higuchi et al., 2002). This suggests that the aforementioned abnormalities alone are insufficient to cause AML. In many of the mouse models there is a long latency of disease, incomplete penetrance, and an acquisition of karyotypic irregularities during progression to AML (Grisolano et al., 1997; Pollock et al., 2001), all indicative of a requirement for secondary genetic and/or epigenetic abnormalities.

1.2.4.2. Activation of Proliferation

Most acute leukemias appear to result from collaboration between abnormalities associated with the impairment of differentiation, and a second class of mutations that confer a proliferative and/or survival advantage to hematopoietic progenitor cells. An example of activating mutations in AML and MDS that confer proliferative advantage are the mutations found in codons 12, 13 and 61 of N-RAS, and codons 12 and 13 of K-RAS (Nakagawa et al., 1992). Given their incidence in preleukemic conditions such as MDS, it is possible that these mutations play a role in the early phases of carcinogenesis. However, the reported incidence of these mutations (25 - 44%) varies greatly between studies (Beaupre and Kurzrock, 1999) and the prognostic value data is conflicting (Neubauer et al., 1994; Ritter et al., 2004). Despite the inconsistent clinical data on RAS mutations, a considerable effort has been devoted to the development of small molecule inhibitors of RAS activation, with a focus on farnesyl transferase and geranylgeranyl transferase (prenyl transferase) inhibitors that prevent targeting of activated RAS to the plasma membrane (Karp, 2001; Sebti and Hamilton, 1997).

Another genetic abnormality associated with leukemic transformation and proliferation is the internal tandem duplication (ITD) of the juxtamembrane domain of the FLT3 gene (Nakao et al., 1996). The ITD is generally monallelic, resulting in a heterozygous increase in FLT3 message length, and is specific for AML and MDS, not being detected in a spectrum of other hematological malignancies and normal hematopoietic cells (Ishii et al., 1999; Yokota et al., 1997). The overall frequency of FLT3-ITD in AML is approximately 24% (Kelly and Gilliland, 2002), occurring in all FAB subtypes, with an incidence that increases with age (Stirewalt et al., 2001). Additionally, mutations in the activation-loop domain of FLT3 have been reported in a small percentage of AML patients with D825Y being the most common amino acid substitution (Yamamoto et al., 2001), making FLT3 the single most commonly mutated gene in AML.

Wild-type FLT3 is a membrane-bound receptor tyrosine kinase and mutations in the juxtamembrane and activation-loop domains are predicted to abolish autoinhibitory activity, resulting in constitutive activation of the FLT3 kinase. ITD mutations of FLT3 result in ligand independent dimerization and tyrosine autophosphorylation (Kiyoi et al., 1998) as well as activation of multiple pathways including STAT5, RAS/MAPK, and PI3K (Hayakawa et al., 2000; Mizuki et al., 2000). Not surprisingly, the recognition of FLT3 mutations has led to the development of several small molecule FLT3 tyrosine kinase inhibitors (Levis and Small, 2004), and although *in vivo* inhibition of FLT3 phosphorylation has been observed (O'Farrell et al., 2003), clinical utility is still under evaluation (Smith et al., 2004).

AML is an aggressive, heterogeneous disease with various cytogenetic abnormalities. As such, the response rates and efficacy of novel FLT3 inhibitors in the treatment of AML patients will most likely not reach those of imatinib mesylate treatment of CML, as the latter disease is uniformly associated with the presence of the BCR/ABL producing t(9;22) translocation. Moreover, in contrast to BCR/ABL, expression of the FLT3-ITD in primary murine hematopoietic progenitors is not sufficient to cause AML (Kelly et al., 2002a), again suggesting that secondary mutations are required for AML development. This hypothesis is supported by the fact that FLT3-ITDs occur concurrently with other previously discussed gene rearrangements and point mutations, which, as mentioned, alone are also insufficient to cause AML. Thus, it is attractive to envision the AML phenotype as a disease that requires collaboration between genetic abnormalities in proliferation and hematopoietic differentiation pathways. This model is likely oversimplified as additional mutations, and epigenetic abnormalities, may play an important role in disease pathogenesis.

Although there are more target genes to be identified, those currently under investigation have provided great insight into the molecular basis of leukemia. Like other cancers, the elucidation of the molecular pathogenesis of AML will undoubtedly improve the design of molecularly targeted therapies for patients with well defined subtypes of disease. Genes involved in important aspects of cell biology that are often targets of deregulation in other cancers will be expanded upon in the following section.

1.3 THE MOLECULAR BASIS OF CANCER

A vast body of research has uncovered many molecular irregularities associated with cancer and as a result, a clear model now exists of how two sets of genes, oncogenes and tumor suppressor genes, participate in cellular transformation. These types of genes have proven to play specific roles in all steps of carcinogenesis and have afforded a foundation for diagnostic and therapeutic development (Weinberg, 1994).

1.3.1. Oncogenes

The first oncogene, a gene that has the potential to cause a normal cell to become cancerous, was identified in 1976 through pioneering work on the Rous sarcoma virus (Stehelin et al., 1976). This gene, identified as SRC, is a proto-oncogene in the human genome whereby retroviral transduction or other *in situ* influence is required to convert it to an oncogene with transforming activity. Examples of *in situ* influence on a proto-oncogene can be categorized into two types of molecular irregularities, changes in the structure of a gene that results in the synthesis of an abnormal oncoprotein, and changes in the expression of a gene that results in the inappropriate production of the normal growth-promoting protein.

A proto-oncogene structure altering mechanism commonly associated with cancer is the introduction of a point mutation that alters the amino acid sequence of the resulting protein. One of the most documented mutational activations associated with a spectrum of human tumors are point mutations within the aforementioned RAS proto-oncogene family (Rodenhuis, 1992). For example,

nearly half of all colon cancers harbor an activating mutation in the K-RAS proto-oncogene, which gives the K-RAS protein potent transforming ability (Bos et al., 1987; Kinzler and Vogelstein, 1996).

Another molecular event that can result in the activation of a proto-oncogene is the chromosomal translocation. As stated earlier, a classic example of a translocation resulting in the formation of a novel oncoprotein is the Philadelphia chromosome, characteristic of CML, which involves the reciprocal genetic exchange between chromosomes 9 and 22 (de Klein et al., 1982; Rowley, 1973). The resulting chimeric protein, BCR/ABL, has potent tyrosine kinase activity and is an effective therapeutic target in myeloid leukemias (Druker, 2004).

Translocation induced overexpression of a proto-oncogene is also demonstrated in Burkitt's lymphoma, where the gene c-MYC, located at 8q24, is juxtaposed to 14q32. This translocation places c-MYC very close to the enhancer elements of the immunoglobulin heavy chain gene, a region with exceptionally high transcriptional activity, and ultimately drives the irregular production of the c-MYC protein product (Gauwerky and Croce, 1993). Interestingly, another member of the MYC family, N-MYC, is an example of increased proto-oncogene expression through a separate mechanism known as gene amplification (Schwab, 1993). This process results from reduplication and amplification of a gene's DNA sequence that can be detected cytogenetically or by fluorescence *in situ* hybridization. In the case of neuroblastoma, an amplified N-MYC gene results in elevated expression of both N-MYC mRNA and protein and is significantly associated with poor prognosis (Schwab, 1993).

However, these types of *in situ* influences on genes throughout the human genome do not always result in the formation of an oncogene. Ultimately, the genetic insults within a cancer cell will often affect a gene whose function is not amenable to assisting cellular transformation and as a result, a formal operational definition of an oncogene was established (Bishop, 1985). If a candidate gene causes the cell to adopt the attributes of a cancer cell (cellular transformation) upon introduction into a normal cell, then the introduced gene can be considered a true oncogene. Functionally, many such oncogene proteins induce cellular transformation by activating growth promoting signaling pathways within the cell in the absence of normal exogenous growth factor stimulation.

1.3.2. Tumor Suppressor Genes

Equally as important as the growth promoting oncogene is the recognition of the growth suppressive, or tumor suppressor gene. The protein products of tumor suppressor genes inhibit the proliferation of a cell and are therefore very important to neoplastic transformation. The retinoblastoma (RB) gene, named after the rare childhood eye tumor in which it was discovered, provides a model for many of these genes (Hollingsworth et al., 1993). Like almost all genes, it is present in two copies per cell. When one of these copies becomes inactivated, cell growth is still normal, in part by relying on the remaining gene copy. Yet, when the second copy is also lost, the growth constraint redundancy is lost and unregulated cellular proliferation proceeds. Importantly, inactivated versions of the RB gene can be passed through the germline from parent to offspring, leaving the

child with a higher probability of accidental loss of the functional RB copy. This concept forms the foundation for the 'two hit' hypothesis postulated by Alfred Knudson in 1971 (Knudson, 1971). As such, through Mendelian laws of inheritance, when particular genetic changes involved in the initiation or progression of a tumor occur in the germ line, all somatic cells of the individual whom inherits this particular change will already carry one of the steps required for malignant change. This results in an increased chance for tumor development and an inherited susceptibility to that particular cancer.

Yet, this dogma is incomplete as tumor suppressor gene function can also be lost by a mechanism distinct from that described for the RB gene. In the case of the p53 tumor suppressor gene, which is mutated in over half of all human cancers (Szymanska and Hainaut, 2003), the initially mutated gene copy loses its growth suppressive function and simultaneously acquires the ability to interfere with the function of the remaining intact copy. This is considered a dominant negative mode of action as the mutant p53 protein compromises the ongoing function of its normal counterpart in the same cell (Blagosklonny, 2000).

With respect to the functional mechanism employed by tumor suppressor genes, the signals and signal transducing pathways are less understood than for those involved in growth promotion. However, analogous to an oncogenes involvement in a growth promoting pathway, tumor suppressor gene proteins are often involved in the receiving or processing of growth inhibitory signals. As such, having lost a tumor suppressor gene and its encoded protein, a cell may then lose its ability to respond to external growth antagonizing signals, such as those

conveyed by the naturally occurring growth suppressive transforming growth factor-β1 (TGF-β1) (Massague, 1990). When certain cells lose RB function, they lose responsiveness to TGF-β1 and continue to grow even when the growth factor is present at relatively high concentrations (Pietenpol et al., 1990), functionally implicating RB in the TGF-β1 pathway. Downstream mediators of the TGF-β1 pathway will be discussed in a subsequent section regarding cell cycle regulation.

1.3.3. Apoptosis Regulation

Cellular transformation may result from activation of oncogenes or inactivation of tumor suppressor genes that are involved in the regulation of apoptosis, or programmed cell death. Dysregulation of apoptosis has been implicated in numerous other pathological conditions, including neurodegenerative diseases and autoimmunity, and in the resistance to cancer therapy (Yu and Zhang, 2004). Studies have revealed that the apoptotic machinery in humans consists of a molecular network of proteins that regulate a cascade of events throughout all the various stages of apoptosis. As such, apoptotic defects can therefore allow neoplastic cells to live beyond their normal lifespan, accumulate genetic mutations, sustain growth under hypoxic conditions, and promote tumor angiogenesis (Hanahan and Weinberg, 2000).

The variety of apoptotic stimuli affecting a cell often signal through the many members of the BCL-2 family of proteins found within the core apoptotic machinery (Yu and Zhang, 2004). Deregulation of this process is observed in follicular B-cell lymphoma where overexpression of the anti-apoptotic BCL-2

occurs at a high frequency due to the gene, located at 18q21, being translocated to the immunoglobulin heavy chain gene on chromosome 14 (Bagg and Cossman, 1992). This process is analogous to the previously mentioned t(8;14) translocation activating the c-MYC oncogene in Burkitt's lymphoma. Conversely, related genes, such as BAX and BAK antagonize the anti-apoptotic BCL-2 and BCL-X_L proteins, and therefore act by initiating or accelerating cell death (Letai et al., 2002). Ultimately, the decision to undergo programmed cell death appears to be based on the relative amounts of pro-apoptotic and anti-apoptotic signals within a given cell, and therefore any gene activating or inactivating irregularity within either pathway will significantly affect the lifespan of that cell.

1.3.4. DNA Repair Regulation

The primary function of the mismatch repair (MMR) system is to eliminate spontaneous single-base mismatches and insertion-deletion loops that arise during DNA replication. These types of errors, if not repaired promptly, can contribute to neoplastic transformation and are therefore vigorously addressed by a family of at least six different eukaryotic MMR proteins (Jiricny and Nystrom-Lahti, 2000). Owing to their importance in maintaining genomic stability, germline alterations in any one of the five human MMR genes, MSH2, MLH1, MSH6, PMS2 and PMS1, gives rise to hereditary nonpolyposis colon cancer (HNPCC) that accounts for 1-5% of all colon cancer cases (Peltomaki, 2001). Predisposed individuals from HNPCC families have a high lifetime risk of developing a range of carcinomas, collectively referred to as the HNPCC tumor spectrum (Aarnio et al., 1999).

Tumors from HNPCC patients often show acquired variation in the number of short repeat units contained within microsatellite sequences, deemed microsatellite instability (MSI), and is the result of a failure to correct repeat errors following DNA replication. Not surprisingly, the mutation rates in tumor cells with MMR deficiency are 100 - 1000 fold greater as compared to normal cells (Bhattacharyya et al., 1994), and can affect important growth regulatory genes including the coding region containing repeats of the TGF-β1 receptor type-II (TGFβRII) gene and the previously mentioned pro-apoptotic BAX gene (Samowitz et al., 2002). Thus, cells lacking a functional MMR system are associated with decreased genomic stability, and if the mutational activity occurs in critical genes involved in growth suppression, apoptosis or signal transduction, a growth advantage may be acquired.

1.3.5. Cell Cycle Regulation

Passage through the eukaryotic cell cycle requires a replication of the genome, doubling of cell mass, a precise segregation of chromosomes and a distribution of other cell components to the daughter cells (Prescott, 1976). The execution of these events divides the cycle into four phases: chromosomes are replicated during the S (synthetic) phase; cellular constituents are divided into daughter cells during the M (mitotic) phase; and two G (gap) phases, G1 and G2, intervene between S and M (Fig. 1.2). In addition, cells can also exit the cell cycle by becoming latent, or quiescent, a period known as G0. Cell growth occurs continuously in G1, S, G2, and M; however it is during G1 and G2 that cells

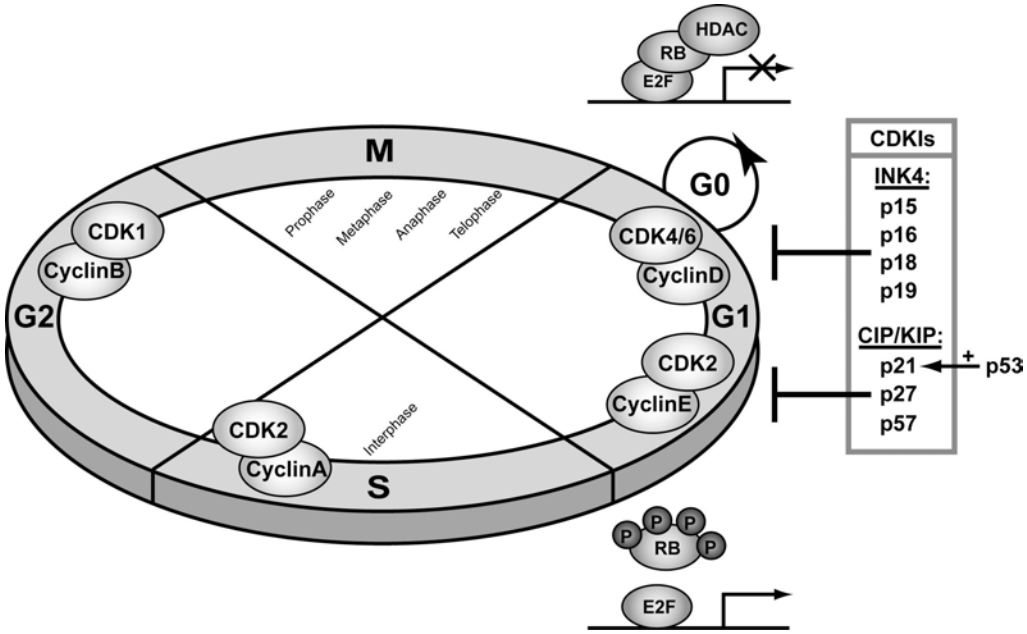

Figure 1.2 Regulation of the Eukaryotic Cell Cycle. Illustrated is the life cycle of a eukaryotic cell that highlights some of the major regulatory features. Progression through the cycle is marked by oscillating levels of a family of proteins known as cyclins, which are regulated, in part, by CDKs. G1 cyclin-CDK complexes are inhibited by two families of listed CDKIs, altogether regulating the phosphorylation status of the RB protein. Unphosphorylated RB binds the E2F transcription factor family and inhibits the transcription of E2F target genes by targeting histone deacetylase activity (Luo et al., 1998), yet when phosphorylated, releases E2F and allows the transcription of multiple E2F target genes. E2F target genes are essential for S phase DNA replication and the G1/S checkpoint is commonly deregulated in cancer.

typically respond to proliferative and antiproliferative signals that determine if the cell cycle ought to proceed. Importantly, the cell cycle has the option of stopping within G1 and G2 without interrupting the critical and precarious events of chromosome replication and chromosome segregation.

Much of what is known about the regulated transition of cells through the cell cycle has come from genetic and biochemical studies carried out in lower organisms. One of the first genes to be recognized as being an important regulator of the cell cycle in yeast was cdc2/cdc28 and, importantly, a regulatory subunit referred to as a 'cyclin' was also identified which is required for cdc2/cdc28 kinase activation (Hartwell, 1978). Cyclins were first identified in marine invertebrates as proteins whose accumulation and degradation oscillated during the cell cycle (Rosenthal et al., 1980). It is the sequential activation and inactivation of cyclin-dependent kinases (CDKs), through the periodic synthesis and destruction of the cyclins, which provide the primary means of cell cycle regulation (Johnson and Walker, 1999). Cyclin expression is rate-limiting for CDK activation, and control of cyclin expression is a fundamental mechanism underlying CDK periodicity. In general, cyclin levels are determined by both transcriptional control and regulated proteolysis by the ubiquitin-proteasome system (Johnson and Walker, 1999).

The primary mammalian cyclins in G1 are the D- and E-type, which associate with the CDK4/6 and CDK2 subunits, respectively. Of note, inhibition of cyclin D1 function blocks the cell cycle in G1, demonstrating the necessity of cyclin D for the cell cycle (Baldin et al., 1993). Progression through the eukaryotic cell cycle, however, is promoted by CDK mediated phosphorylation of protein

substrates, most notably RB (Weinberg, 1995). The RB protein is crucial for control of the G1/S checkpoint within the cell cycle and, as mentioned, the RB gene is frequently inactivated in cancer cells. Normal passage through G1/S of the cell cycle occurs when the RB protein undergoes cell cycle-dependent phosphorylation during G1, disrupting its interaction with the essential E2F family of transcription factors (Buchkovich et al., 1989). E2F factors regulate the expression of many genes that encode proteins involved in cell cycle progression and DNA synthesis but when complexed with an unphosphorylated RB, E2F is inactive and the cell cycle arrests in the absence of these much needed gene products. However, as cycling cells progress through G1, RB is progressively phosphorylated by CDKs at multiple sites, ultimately releasing E2F and allowing transcription of essential S phase genes.

1.3.6. Cyclin Dependent Kinase Inhibitors

All organisms express proteins that directly bind to and inhibit CDK activity (Sherr and Roberts, 1995) (Fig. 1.2). These cyclin dependent kinase inhibitors (CDKIs) provide another important mechanism by which CDK activity is regulated in response to diverse stimuli. Mammalian cells express two classes of CDKIs that are distinguished by their CDK targets: the CIP/KIP family of CDKIs, which are universal CDK inhibitors, and the INK4 CDKIs, which are specific for CDK4/6 inhibition (Sherr and Roberts, 1995).

1.3.6.1. The CIP/KIP CDKI Family

The CIP/KIP family of CDKIs consists of three members: p21CIP1/WAF1 (p21), p27KIP1 (p27), and p57KIP2 (p57). Overexpression of these molecules causes a G1 arrest in cultured cells and they are able to inhibit most cyclin-CDK complexes in proliferating cells *in vitro* (Sherr and Roberts, 1995). The first of this family to be isolated was p21 which was identified by several independent laboratories employing a variety of strategies (el-Deiry et al., 1993; Harper et al., 1993; Xiong et al., 1993). As illustrated in Figure 1.3, the p21 protein contains two functional domains, an amino-terminal CDK interaction region that is sufficient for CDK inhibition, and a carboxy-terminal region that binds proliferating cell nuclear antigen (PCNA), a processivity factor associated with DNA polymerase-δ (Waga et al., 1994). Thus, induction of p21, which can occur in response to DNA damage through a p53-dependent mechanism (el-Deiry et al., 1993), contributes to cell cycle arrest in two ways: by inhibiting cyclin-CDK complexes, and by inhibiting DNA synthesis through PCNA binding.

The CDKIs p27 and p57 are structurally related to p21 as they share significant amino-terminal homology within the CDK inhibitory domain, but p27 and p57 do not contain the PCNA interaction region (Lee et al., 1995; Polyak et al., 1994) (Fig. 1.3). Both p27 and p57 bind to a variety of cyclin-CDK complexes, yet only p27 has been implicated in modulation of contact inhibition (Polyak et al., 1994). Furthermore, like p21, p27 also mediates several growth inhibitory signals including the aforementioned growth factor TGF-β1 (Robson et al., 1999). In contrast to the ubiquitous expression of p21 and p27, p57 displays a tissue-specific

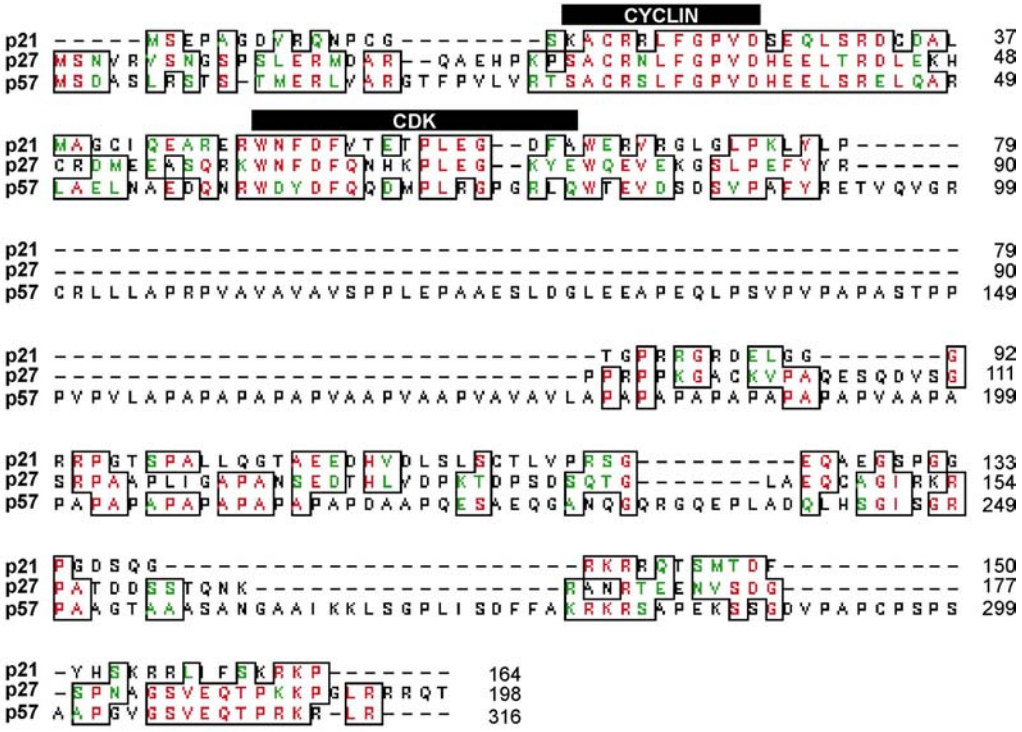

Figure 1.3 CIP/KIP CDKI Proteins. Illustrated are the amino acid alignments of CDKIs p21, p27, and p57 revealing the common cyclin and CDK binding domains (represented by black bars above sequence). The carboxy-terminal PCNA interaction region of p21 is not shown and numbers represent amino acid position. Alignments were generated using the 'ClustalW' amino acid sequence alignment algorithm and the 'prettyplot' diagram tool available from the European Molecular Biology Open Software Suite (EMBOSS).

expression pattern suggesting a specialized role in cell cycle control. A role for p57 in carcinogenesis is evidenced by the fact that the p57 gene locus is subject to imprinting, with preferential expression of the maternal allele that is associated with various cancers (Kondo et al., 1996). Taken together, as essential inhibitory mediators of the G1/S cell cycle checkpoint, and therefore cellular proliferation, all three members of the CIP/KIP family of CDKIs are associated with cellular transformation by unique mechanisms (Lee and Yang, 2001).

1.3.6.2. The INK4 CDKI Family

The second family of CDKIs is the INK4 family, consisting of p16INK4a (p16), p15INK4b (p15), p18INK4c (p18), and p19INK4d (p19). The majority of the INK4 proteins, when bound to CDKs, prevents the association of CDK4 and CDK6 with the D-type cyclins and can also inhibit the activity of preassembled cyclin D-CDK4/6 complexes. This activity is accomplished through homologous tandem ankyrin repeats (Sherr and Roberts, 1995), which are motifs of about 34 amino acids and are often involved in protein-protein interaction (Bork, 1993). As illustrated in Figure 1.4, p16 and p15 contain four ankyrin repeats (ankyrin I-IV), while p18 and p19 contain five (ankyrin I-V).

Like RB, the p16 gene is altered in a high percentage of human tumors by a variety of mechanisms including deletion, point mutation, and promoter hypermethylation, an epigenetic mechanism (Kamb et al., 1994; Okamoto et al., 1994). Interestingly, analysis of p16 is complicated in the fact that its 9p21 locus encodes a second protein in an overlapping alternative reading frame (ARF)

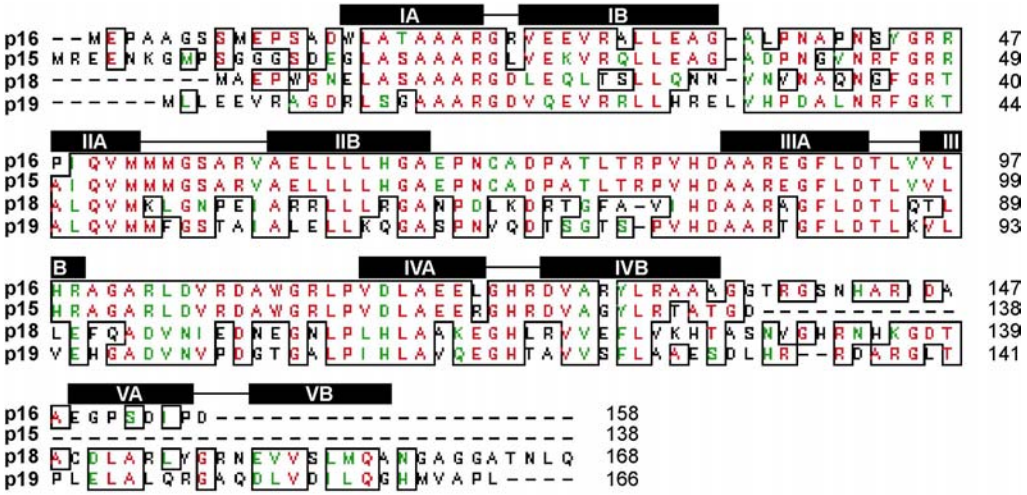

Figure 1.4 INK4 CDKI Proteins. Illustrated are the amino acid alignments of CDKIs p16, p15, p18, and p19 revealing the common ankyrin repeat protein binding domains (represented by black bars above sequence). Numbers represent amino acid position. Alignments were generated using the 'ClustalW' amino acid sequence alignment algorithm and the 'prettyplot' diagram tool available from EMBOSS.

(Quelle et al., 1995) (Fig. 1.5). This non-INK4 family alternative reading frame protein (p14ARF) has been shown to assist in the regulation of p53 stability by counteracting the MDM2-mediated destruction of p53 (Bothner et al., 2001; Zhang et al., 1998). Furthermore, in many tumors and cell lines with p16 deletion, the related and closely linked p15 gene is also affected (Sherr, 1996). However, unlike p16, p15 is unaffected by RB status but can, like p21 and p27, be induced by TGF-β1 (Hannon and Beach, 1994). The frequent deletions of p15 and p16 in primary tumors and the high spontaneous tumor rate in p16-deficient mice indicate that these proteins play a critical role in maintaining normal growth control (Serrano et al., 1996).

Of note, the INK4 family member p18, located at 1p32, which has largely been considered to be unaffected in human tumors (Otsuki et al., 1996), has recently been shown to be silenced by promoter hypermethylation in Hodgkin lymphomas (Sanchez-Aguilera et al., 2004). Similarly, the INK4 family member p19, located at 19p13, has recently been associated with aberrant epigenetic transcriptional silencing. The gene, previously reported to lack inactivating mutations in cell lines and primary tumors (Zariwala and Xiong, 1996), has been shown to be upregulated following treatment with the HDAC inhibitor trichostatin A (TSA), implicating epigenetic silencing at this locus in the human T-cell leukemia cell line Jurkat (Yokota et al., 2004).

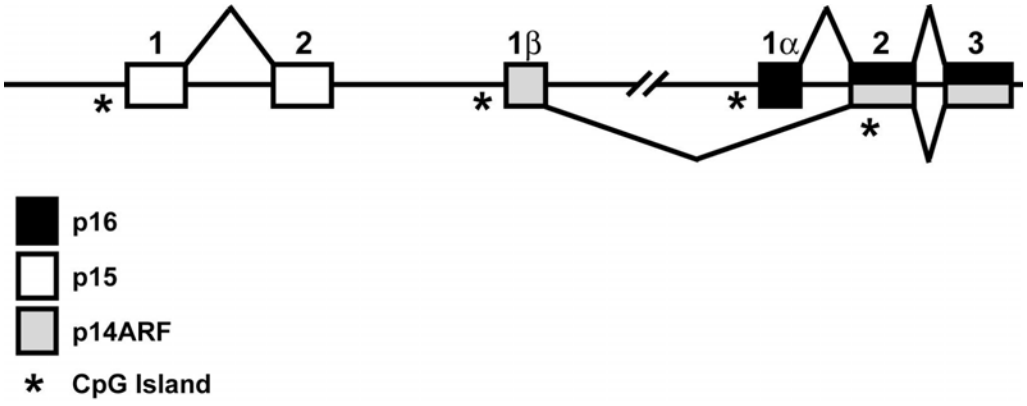

Figure 1.5 INK4 Genomic Locus at 9p21. The genomic organization at 9p21 houses two members of the INK4 family of CDKIs, p15 and p16, and an unrelated gene p14ARF. Interestingly, p14ARF utilizes two of the same exons as p16 but is translated in an alternative reading frame. Boxes represent exons and CpG islands (described in Section 1.4.1.) are represented by asterisks.

1.3.7. Multistep Molecular Carcinogenesis

In 1990, Fearon and Vogelstein proposed a highly publicized genetic model of a multistep pathway for colorectal tumorigenesis (Fearon and Vogelstein, 1990). The model illustrates the concept that as a cell evolves through the steps of transformation, including hyperplasia, metaplasia, neoplasia, and metastasis, that multiple genetic 'hits' are acquired affecting genes in several pathways. As such, genetic abnormalities are reflected histologically by precursor lesions that progress to common features of malignancy. Specifically, the model predicts inactivation of the adenomatous polyposis coli (APC) gene, a common mutation in both sporadic and familial colorectal cancer (Tomlinson et al., 1997), as an early event in the formation of a small benign adenoma. Other genetic insults purported to be important in cell transformation include RAS proto-oncogene activation and loss of the deleted in colorectal cancer (DCC) gene. Loss of p53 is predicted as a late adenoma evolves into a malignant cancer, or carcinoma, at which point the model recognizes other, as yet unidentified, alterations during the final progression to an invasive and metastatic tumor.

Although the multistep model of carcinogenesis is widely accepted for colorectal cancer and other common human tumors, it has since been modified to account for novel genes and recently described mechanisms of molecular aberrancy, including epigenetic mechanisms (Bellacosa, 2003; Ilyas et al., 1999; Issa, 2000; Kinzler and Vogelstein, 1996). As such, the basic model is admittedly oversimplified, most likely underestimating the number of genes involved in clonal transformation, yet it has provided a resilient framework for the continued

understanding of the molecular basis of cancer, specifically that of colorectal cancer. Identifying all the molecular irregularities, both genetic and epigenetic, and the interactions of the genes involved therein will undoubtedly assist in further understanding how a tumor evolves, potential points of intervention, and prognostic implications of the molecular abnormalities. Furthermore, transposing this model to leukemia and identifying novel genes involved in proliferation and impaired differentiation could assist the development of targeted therapies. The concept of epigenetics and its involvement in cancer biology is expanded upon further in the subsequent section.

1.4 EPIGENETICS

The term ‘epigenetics’ was originally defined in the 1940s by the developmental biologist Conrad H. Waddington and, since then, has been used in a variety of forms. In its original definition it was a manifestation of two concepts, ‘epigenesis’ and ‘genetics’ (Van Speybroeck, 2002), and has since been the topic of much philosophical debate over its precise meaning (Jablonka and Lamb, 2002). Regardless of its many forms, the term ‘epigenetics’ largely involves two major concepts: 1) the study of heritable developmental processes within an organism, and 2) the study of heritable changes in gene expression that occur without a change in the genomic DNA sequence. In both instances the major focus is how gene regulatory information that is not encoded in the DNA sequence is transmitted from one generation, either of cells or organisms, to the next. Studies on DNA methylation and chromatin structure, which became increasingly prominent in the 1960s and 1970s, identified molecular systems that account for epigenetic variation and have since become the focal point of contemporary epigenetic theory (Henikoff and Matzke, 1997).

1.4.1. DNA Methylation

DNA methylation in higher eukaryotes affects only the pyrimidine base cytosine which becomes covalently modified at the carbon atom five position (Fig. 1.6). This modification only takes place when a cytosine is followed by a guanine base, and therefore only the dinucleotide CpG is methylated throughout the mammalian genome. Interestingly, methylated cytosine is a target for point

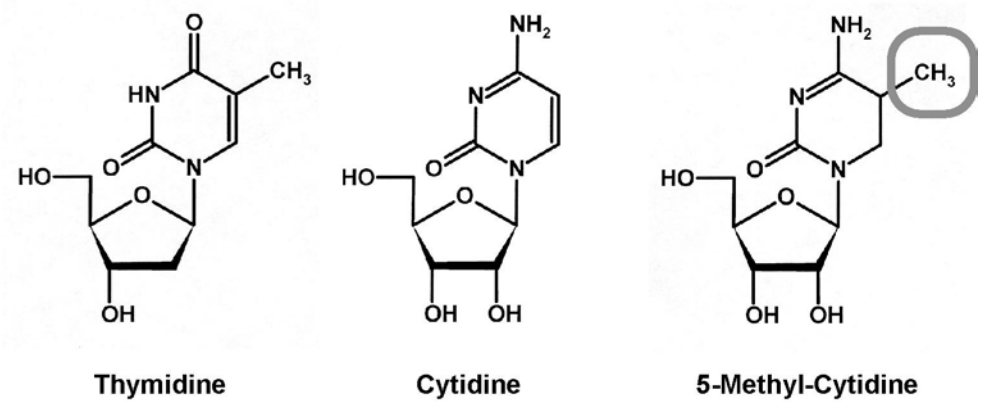

Figure 1.6 Pyrimidine Nucleoside and 5-Methyl-Cytidine Chemical Structures. Illustrated are the chemical structures and names of the common pyrimidine nucleosides, thymidine and cytidine, and the modified 5-methyl-cytidine. The grey box highlights the methyl group attached to the carbon-5 of cytidine. Structures were constructed using ChemSketch Version 5.12 software (Advanced Chemistry Development Inc., Toronto, ON, Canada).

mutation as deamination of 5-methyl-cytidine leads to the formation of the naturally occurring DNA base thymidine, which is not recognized as mutated by the DNA repair machinery (Rideout et al., 1990). As deamination is constantly taking place under physiological conditions, there is a resulting selective pressure that reduces the number of methylated cytosine bases within the genome. In the mammalian genome, this has led to a progressive depletion of the CpG dinucleotide throughout evolution (Cooper and Krawczak, 1989).

Only the physiologically unmethylated CpG sites in the promoter region and first exons of transcribed genes remain unaltered in part because active transcription seems to protect CpG sites against methylation (Clark and Melki, 2002). These typically unmethylated CpG-rich stretches of DNA form 'CpG islands' throughout the vast CpG depleted genome. CpG islands comprise 1 - 2% of the genome and were originally arbitrarily characterized by an overall GC content of greater than 50% and an approximately five times more frequent occurrence of the dinucleotide CpG (Gardiner-Garden and Frommer, 1987). In order to avoid many of the recently identified GC-rich sequences not associated with promoter regions, such as Alu-repetitive elements (approximately 0.3 kb in size) and intragenomic parasites, the CpG island definition has recently been modified with slightly more stringent criteria (Takai and Jones, 2002). Notable exceptions to the unmethylated CpG island dogma are the constitutively methylated CpG islands found within imprinted genes (Reik and Walter, 2001) and those found on the inactive X-chromosome in females (Heard, 2004). These concepts will be discussed in more detail in subsequent sections.

1.4.1.1. Global Hypomethylation

A global loss of DNA methylation at CpG dinucleotides was the first epigenetic abnormality to be identified in cancer cells (Feinberg and Vogelstein, 1983a). In this study, utilizing methylation sensitive restriction enzymes and southern blotting, the authors found that a substantial proportion of CpGs that were methylated in normal tissues were unmethylated in cancer cells, which was later confirmed that same year using high-performance liquid chromatography (Gama-Sosa et al., 1983). Since then, global deficiencies in methylated cytosine content have been observed in prostatic tumors (Bedford and van Helden, 1987), leukemias (Wahlfors et al., 1992), hepatocellular carcinomas (Lin et al., 2001), cervical cancers (Kim et al., 1994), and breast ductal carcinomas (Bernardino et al., 1997), despite the frequent finding of localized hypermethylation. The biological significance of DNA hypomethylation in cancer is still not fully understood, however the high frequency of cancer-associated DNA hypomethylation, the nature of the affected sequences, and the absence of any association with regional DNA hypermethylation are consistent with an independent role in assisting cancer formation and tumor progression.

Hypomethylation observed in cancer is often found in DNA repeats, most notably endogenous retrotransposons, which tend to be highly methylated in somatic tissues (Ehrlich, 2002). For example, *in vivo* hypomethylation dependent transcriptional upregulation of the repetitive retrotransposon LINE-1 has been shown to interfere with karyotypic stability and the transcriptional activity of neighboring genes, ultimately affecting phenotype and disease risk (Whitelaw and

Martin, 2001). Furthermore, the normally methylated small Alu-repetitive elements, which constitute approximately 10% of the genome, can be transcribed and mobilized by tumor associated DNA hypomethylation, occasionally leading to cancer-associated gene insertions (Liu et al., 1994). Another class of methylated human retrotransposons that become hypomethylated in association with oncogenesis are endogenous retroviruses, especially those from the HERV-K family (Florl et al., 1999). It is speculated that decreased methylation of the HERV-K retroelement, like that of Alu-repeats and LINE-1, may contribute to genomic instability in specific human tumors by rendering these normally repressed sequences competent for transcription and recombination.

Interestingly, it has been reported that a limited number of proto-oncogene associated CpG islands are methylated in somatic tissues and that cancer associated hypomethylation can actually lead to gene activation (Feinberg and Vogelstein, 1983b; Strichman-Almashanu et al., 2002). Strong support for hypomethylation leading to activation of cancer related genes comes from reports linking the overexpression of cyclin D2 in gastric carcinoma (Oshimo et al., 2003), the S100A4 metastasis-associated gene in colon cancer (Nakamura and Takenaga, 1998), and the 14-3-3σ proliferation-associated gene in pancreatic cancer (Sato et al., 2003b). Moreover, hypomethylation of the multidrug-resistance gene (MDR1) has been identified in AML and is strongly associated with increased aberrant MDR1 expression, the acquisition of drug resistance, and adverse clinical outcome (Nakayama et al., 1998).

Taken together, while there is only a limited understanding of the pathways that lead to cancer-associated hypomethylation of some sequences and hypermethylation of others, there are some clues as to the types of sequences generally affected. As mentioned, hypomethylation in repetitive DNA sequences may play a special role in carcinogenesis, such as increasing karyotypic instability or indirectly affecting gene expression, and evidence does exist for direct expression linked hypomethylation of cancer related genes. For some types of cancer, DNA hypomethylation is seen as an early indicator of tumorigenesis (Feinberg et al., 1988) and therefore may have the potential to become a clinically useful diagnostic aid. Although DNA hypomethylation is often overlooked when studying the relationship between methylation and carcinogenesis, its contribution to cell transformation may in the future be considered equivalent to that of the phenomenon of DNA hypermethylation.

1.4.1.2. CpG Island Hypermethylation

Site specific DNA hypermethylation was first reported in 1986 by Stephen Baylin and colleagues who described 5'-promoter region CpG methylation of the calcitonin gene in association with transcriptional silencing in human lung cancers and lymphomas (Baylin et al., 1986). Although the function of the calcitonin gene has yet to be directly related to carcinogenesis, it was the first study to show cancer associated site specific DNA hypermethylation as compared to that of normal adult tissue. It was not until 1989 when hypermethylation associated transcriptional silencing of a bona fide tumor suppressor gene was reported in cancer, that of the

RB gene in retinoblastoma (Greger et al., 1989). This important finding was the first to establish aberrant epigenetics, as opposed to loss-of-function mutations, as a novel mechanism behind disease associated gene silencing. This finding was later confirmed by the identification of allele specific hypermethylation associated inactivation of the gene in primary retinoblastomas (Sakai et al., 1991). Mechanistic analysis of the RB promoter came in 1993 when Ohtani-Fujita et al. reported that hypermethylation of the RB promoter *in vitro* reduced the gene's level of expression to 8% of the unmethylated control and that essential transcription factors could not bind their RB promoter recognition sequences when the CpGs within were methylated (Ohtani-Fujita et al., 1993). Based on these observations, the mechanism behind promoter hypermethylation mediated gene silencing was long considered to be solely based on the steric blockage of transcription factor binding.

The subsequent few years following the discovery of RB promoter hypermethylation resulted in a marked increase in research that identified numerous loci in cancer cells which are targets for hypermethylation associated transcriptional silencing. Key tumor suppressor genes, including p16 (Gonzalez-Zulueta et al., 1995), MLH1 (Kane et al., 1997), the von Huppel-Lindau (VHL) gene (Herman et al., 1994), and E-cadherin (Graff et al., 1995), have all been shown to be repressed both in cell lines and in primary cancers by an epigenetic mechanism that correlates with dense promoter associated CpG island hypermethylation. As such, evidence has accumulated implicating CpG island hypermethylation as a novel mechanism of transcriptional silencing that can affect

multiple pathways involved in various components of cellular biology, including cell cycle regulation, apoptosis, mismatch repair, and contact inhibition.

1.4.1.3. Genomic Imprinting in Human Cancer

Genomic imprinting is the epigenetic marking of a gene or genes based on its parental origin that results in monoallelic expression, despite both parents contributing equally to the genetic content of their progeny. The mechanism of imprinting is complex and not fully understood, yet prevailing evidence implicates parental-specific methylation of CpG-rich domains which are likely established during gametogenesis (Ferguson-Smith et al., 1993). Imprinted genes are involved in many aspects of development including fetal and placental growth, cell proliferation, and adult behavior. It is therefore not surprising to presuppose that, when altered, imprinted genes are often involved in numerous human diseases including cancer (Falls et al., 1999).

Imprinted genes can be involved in carcinogenesis in several ways. For example, loss of heterozygosity (LOH) at an imprinted region may result in the deletion of the only functional copy of a tumor suppressor gene and conversely, loss of imprinting (LOI) of an imprinted proto-oncogene may allow for inappropriate increases in gene expression (Feinberg, 1993). The most common example of aberrant genomic imprinting associated with cancer involves the childhood Wilms' tumor and the reported high frequency of irregular biallelic insulin-like growth factor 2 (IGF2) expression (Ogawa et al., 1993). Interestingly, LOI of IGF2, located at 11p15.5, has been linked to LOH of the neighboring H19

gene in this disease, resulting in a complex enhancer competition model for the reciprocal control of expression between the two genes (Webber et al., 1998). The model involves a 2.4 kb imprinting control region regulating the somatic monoallelic expression of IGF2 and H19, which is achieved through a DNA methylation dependent chromatin insulator (known as CTCF) and promoter silencing activities on the maternal and paternal alleles, respectively (Webber et al., 1998). Of note, many adult tumors have since showed LOI at the IGF2 locus, indicating aberrant IGF2 imprinting in the pathogenesis of a variety of cancers.

As previously mentioned, the p57 gene, also located at 11p15.5, is a maternally expressed and paternally imprinted CDKI and, owing to its location, biochemical functions and imprinting status, is considered a candidate tumor suppressor gene (Kondo et al., 1996). As a candidate tumor suppressor gene, LOH of the remaining expressed p57 copy, through either genetic or epigenetic means, could hypothetically assist cellular transformation and its reduced expression in human Wilms' tumors and Beckwith-Wiedemann syndrome (BWS) supports this notion (Thompson et al., 1996). Genetic analysis in BWS, a prenatal cancer-predisposing disorder, has revealed missense and frameshift mutations of the maternal p57 allele in a significant number of cases resulting in little or no active p57 protein (Hatada et al., 1996). Yet, the role of DNA methylation in the control of p57 imprinting has been questioned as evidence has shown that p57 is expressed in nonneoplastic tissues in the absence of promoter hypermethylation.

The methylation status of the p57 promoter associated CpG island has been recently evaluated in many tumor cell lines and primary cancers and a high

frequency of cases harbored promoter hypermethylation in association with decreased p57 mRNA expression (Kikuchi et al., 2002; Li et al., 2002b; Shen et al., 2003). Importantly, treatment of methylated ALL cell lines with 5-Aza-2'-deoxycytidine (5-Aza-dC), a DNA methyltransferase inhibitor, restored p57 expression in association with CpG island demethylation. Moreover, in one of these studies deacetylated histones H3 (H3) and H4 (H4) were found to be associated with methylated p57 promoter alleles (Kikuchi et al., 2002), implicating a functional role for histone modifications and chromatin conformation in promoter hypermethylation mediated gene silencing. Although the role of DNA hypermethylation in p57 genomic imprinting is as yet unresolved, evidence is accumulating implicating p57 promoter specific hypermethylation in many hematological malignancies.

1.4.1.4. Mammalian DNA Methyltransferases

Cytosine methylation, as stated, is known to be involved in transposon and other repetitive element silencing, gene specific transcriptional regulation, and genomic imprinting. In addition, recent evidence supports a role for DNA methylation, as well as unique RNA coating and chromatin remodeling, in the developmental silencing of multiple genes located on one of the two X-chromosomes in XX female mammals (Heard, 2004). Although it is still unknown what signals or triggers mammalian methylation, what is consistent throughout these processes is the DNA methyltransferase (DNMT) enzyme family involved therein.

The methyl donor for cytosine methylation is the cofactor *S*-adenosyl-L-methionine and the catalytic reaction involves the target cytosine to become completely everted from the DNA helix and inserted deep into the active site of the DNMT enzyme (Bestor, 2000). In mammals, three families of DNA cytosine-5 methyltransferase enzymes, DNMT1, DNMT2, DNMT3a, and DNMT3b, have been identified. DNMT1 is the largest methyltransferase with a molecular mass of 184 kDa (Smith et al., 1992). The amino-terminal two-thirds of the protein is considered to be the regulatory domain and the carboxy-terminal region contains the catalytic domain (Yen et al., 1992), which is homologous with all the known DNMTs (Kumar et al., 1994). In proliferating cells, DNMT1 is found to be associated with replication foci (Leonhardt et al., 1992), ensuring reciprocal methylation of the newly synthesized daughter strand during replication, a process known as maintenance methylation. Importantly, disruption of DNMT1 in mice results in abnormal imprinting (Li et al., 1993), embryonic lethality, greatly reduced levels of DNA methylation (Li et al., 1992) and derepression of endogenous retroviruses (Walsh et al., 1998), further supporting the important role epigenetics plays in cell biology and development. In comparison with DNMT1, DNMT2 is much smaller, with a predicted molecular mass of 45 kDa, lacking the large amino-terminal, but containing all the conserved methyltransferase motifs. Biological activity for this protein has yet to be demonstrated and is as yet unknown.

The discovery of the third family of methyltransferases, DNMT3a and DNMT3b, confirmed the presence of *de novo* methylation in mammals (Okano et

al., 1998). Both of these enzymes are crucial for embryonic development and are responsible for the *de novo* methylation during embryogenesis that establishes the somatic methylation pattern of the organism (Okano et al., 1999). DNMT3a and DNMT3b are intermediate in size (100 - 130 kDa) in comparison with DNMT1 and DNMT2, and possess a smaller amino-terminal region. Mutation within the carboxy-terminal of human DNMT3b has been shown to be associated with ICF (immunodeficiency, centromeric instability and facial anomalies) syndrome, characterized by abnormally hypomethylated centromeric satellite sequences and genomic instability (Xu et al., 1999).

It is commonly hypothesized that maintenance and *de novo* methylation must be performed by separate enzymes. As such, sequence specific *de novo* methyltransferases act at specific stages of gametogenesis and early development to establish the methylation patterns, which are then maintained during cell division by sequence independent DNMTs that methylate only hemimethylated substrates (Bestor, 2000). In this model DNMT3a and DNMT3b act as the *de novo* enzymes and DNMT1 is responsible for methylation maintenance (Okano et al., 1999). However, many facets of this paradigm have yet to be fully proven; for example, no mammalian DNMT has been shown to be sequence specific and recent evidence actually supports a role for cooperation between DNMT1 and DNMT3b in the methylation dependent silencing of genes in cancer cells (Rhee et al., 2002). Although the exact responsibilities, either independent or redundant, of mammalian DNMTs have yet to be fully elucidated, there is no question regarding their important roles in development, imprinting and transcriptional regulation, and their

association with various disease states. Furthermore, it should be noted that novel as yet undiscovered mammalian DNMTs may still exist.

1.4.1.5. Mammalian Methyl-CpG Binding Domain Proteins

Once established, the DNA methylation profile of tumor cells is unlikely to be the primary event in gene inactivation. Rather, DNA hypermethylation is thought to provide a long term and robust memory for 'locking in' a transcriptionally inactive state established, in part, by other mechanisms (Bird, 2002). Part of the machinery involved in hypermethylation mediated gene silencing involves a family of proteins that contain a conserved methyl-CpG binding domain (MBD) (Hendrich and Bird, 1998). There are presently five known mammalian MBD proteins in which four, MeCP2, MBD1, MBD2, and MBD4, have the capacity to specifically target methylated CpG dinucleotides through the MBD motif. Like these four proteins, the related MBD3 protein contains this highly conserved MBD motif but, despite having over 70% amino acid similarity to the MBD2 protein, has lost the ability to specifically bind methylated DNA (Hendrich and Bird, 1998). The fifth MBD protein that targets methylated CpG dinucleotides, KAISO, is different from the others in that it binds methylated DNA through a zinc finger motif (Prokhortchouk et al., 2001). Interestingly, unlike the other family members, MBD4 appears to not be involved in hypermethylation mediated transcriptional repression but rather DNA repair by interacting with the previously mentioned MMR protein MLH1 (Bellacosa et al., 1999). Due to the preference for MBD4 to bind 5-methyl-CpG-to-TpG

mismatches, the previously mentioned product of 5-methyl-cytidine deamination, it has been proposed that the role of MBD4 is to minimize mutation at 5-methyl-CpG (Hendrich et al., 1999b), and of note, when removed from mice, is associated with significantly increased CpG mutability and tumorigenesis (Millar et al., 2002).

The first MBD protein to be cloned was the second methyl-CpG binding activity to be discovered, accordingly named MeCP2 (Lewis et al., 1992), and was the protein in which the MBD was defined (Nan et al., 1993). Located at Xq28, it is a highly abundant chromosomal protein that colocalizes in the nucleus with methylated DNA, suggesting a role as a global transcriptional repressor in vertebrate genomes (Nan et al., 1997). Mutations in the MeCP2 gene cause human Rett syndrome, which is a progressive neurologic developmental disorder primarily affecting females (Amir et al., 1999). Those suffering from Rett syndrome, one of the most common causes of mental retardation in females, are actually heterozygous for MeCP2 inactivating mutations. In this instance, aberrant inactivation of the single wild-type MeCP2 allele, located on the X chromosome, abrogates MeCP2 protein function and contributes to neuronal degeneration.

MBD1 is unique among the MBD proteins in that it is capable of repressing transcription from both methylated and unmethylated promoters in cell transfection experiments (Fujita et al., 1999). MBD1 is also unique among the methyl-CpG binding repressors in that it contains within it multiple copies of a cysteine-rich motif (CXXC) with unknown function that is also found in DNMT1, the histone methyltransferase (HMT) MLL, and numerous other uncharacterized expressed sequence tags (ESTs) (Fujita et al., 1999). Similar to MeCP2, MBD1 is an

abundant chromosomal protein (Ng et al., 2000), which contains a powerful transcriptional repression domain (Fujita et al., 2000; Ng et al., 2000), yet a proportion of its repressional activity appears to rely upon the recruitment of HDAC activity (Patra et al., 2003), a concept that will be elaborated on in subsequent sections.

MBD2 and MBD3 are the only vertebrate MBD proteins for which homologues can be identified in invertebrate genomes or EST collections, and thus, MBD2/3 is predicted to represent the original MBD protein (Hendrich et al., 1999a), likely divided into two genes by an evolutionary gene duplication event. Although MBD3 does not have methyl-CpG binding capacity, it is part of an abundant nucleosome remodeling and histone deacetylation (NuRD) co-repressor complex in humans (Zhang et al., 1999), which can be recruited to DNA by several different repressor proteins and is important in embryonic development (Ahringer, 2000). MBD2 is a component of the methyl-CpG binding protein 1 (MeCP1) complex (Ng et al., 1999), which was the first methyl-CpG binding activity to be described, repressing transcription in a methylation density-dependent fashion (Bird and Wolffe, 1999). Interestingly, biochemical purification in HeLa cells has revealed that MeCP1 actually consists of the NuRD complex in association with MBD2 (Feng and Zhang, 2001), substantiating the close relationship between MBD2 and MBD3.

Molecular evidence for the importance of MBD proteins in control of gene transcription *in vivo* has been illustrated by chromatin immunoprecipitation assays in hepatocellular carcinoma (Bakker et al., 2002), colon cancer (Magdinier and

Wolffe, 2001), bladder cancer (Nguyen et al., 2002), and T-cell leukemia cell lines (El-Osta et al., 2002). These studies demonstrate the physical presence of MBD proteins, most often MeCP2 and/or MBD2, at several hypermethylated tumor suppressor gene promoters and further confirm the association between DNA hypermethylation and histone deacetylation. Importantly, treatment with 5-Aza-dC results in predicted promoter demethylation and the release of MBD proteins at p16 (Magdinier and Wolffe, 2001; Nguyen et al., 2002) and MDR1 promoter loci (El-Osta et al., 2002) in association with local enrichment of H3 and H4 acetylation. Thus, the recruitment of MBD proteins to hypermethylated tumor suppressor gene promoters supports a model that involves another epigenetic process, that of chromatin remodeling and post-translational histone modification, which directs transcriptional silencing by means of a repressive chromatin conformation.

1.4.2. Post-translational Histone Modification

Eukaryotic chromosomes, which consist of a highly ordered and condensed protein:DNA complex known as chromatin, are dynamic entities whose appearance varies with the stage of the cell cycle. Individual chromosomes assume their familiar condensed forms only during M phase of the cell cycle, yet during the remainder of the cell cycle, when the chromosomal DNA is transcribed and replicated, the chromosomes of most cells become highly dispersed and indistinguishable. As a result, two different forms of chromatin have been defined: 'heterochromatin', which is tightly compacted and associated with transcriptionally

silent genomic regions, and 'euchromatin', which has a more open or relaxed conformation that tends to support transcription (Wolffe and Kurumizaka, 1998).

1.4.2.1. Nucleosomes and Chromatin Organization

The protein component of chromatin, which comprises over half of the total chromatin mass, consists primarily of histones. All histone proteins have a large proportion of positively charged arginine and lysine residues (Smith, 1991) and consist of five families: histone H1 (H1), H2A (H2A) and H2B (H2B), and the aforementioned H3 and H4. The fundamental unit of the chromatin polymer is the nucleosome, identified in 1974, which consists of approximately 146 base pairs of DNA wrapped around an octamer of core histones, comprised of two copies each of H2A, H2B, H3 and H4 (Kornberg, 1974). Core histones are evolutionarily conserved and consist of flexible amino-terminal 'tails' protruding outward from the nucleosome, and globular carboxy-terminal domains making up the nucleosome scaffold. These positively charged proteins ionically bind DNA's negatively charged phosphate groups, allowing the DNA to form two complete left-handed superhelical turns around the octamer. The linker H1 is responsible for binding the nucleosomal DNA in a cavity of the core particle where DNA both enters and exits the nucleosome, which creates a slightly larger histone:DNA structure known as the chromatosome (Allan et al., 1980).

Nucleosomes form *in vivo* at physiological salt concentrations with the assistance of molecular chaperones such as nucleoplasmin and DNA topoisomerase I, which assist in bringing histones and DNA together in a controlled fashion

(Lilley and Pardon, 1979). The next level of chromosomal organization is achieved through contacts between multiple nucleosomal H1 proteins forming a 300Å-thick filament (Allan et al., 1980). Although little is known how the filaments undergo higher order organization to form functional metaphase chromosomes, it is believed that multiple filaments are packaged along a scaffolding fibrous protein forming a solenoid, and eventually a comprehensive chromosome.

As mentioned, following cell division chromosomes unravel into long thin extended microfibrils of euchromatin or condensed coiled masses of heterochromatin. In order to successfully regulate gene transcription, euchromatic regions require several proteins and multisubunit complexes to act directly on chromatin structure. Aside from transcription, other processes such as chromosomal segregation, replication, recombination and repair also require chromatin manipulation and it has recently become clear that covalent modification of regional histones facilitates this process. Evidence for this stems from the many reports correlating chromatin activity with specific post-translational modifications of the 25 - 40 amino acid residues within the flexible histone amino-terminal tails (Luo and Dean, 1999; Strahl et al., 1999).

By interacting with other chromatin remodeling factors the histone tails themselves therefore provide additional gene regulatory information that contributes to chromatin conformation and is the foundation for the recently hypothesized 'histone code' (Jenuwein and Allis, 2001; Strahl and Allis, 2000). This popular hypothesis predicts that distinct histone modifications act sequentially or in combination to form a code that is read by other proteins to bring about

distinct downstream events. These downstream events include modulation of transcription and the post-translational modifications at the center of this paradigm include histone acetylation, phosphorylation, methylation, ubiquitination, and ADP-ribosylation (van Holde, 1988).

1.4.2.2. Histone Acetylation

Acetylation and deacetylation of the ε-amino groups of conserved lysine residues present in histone tails have long been linked to transcriptional activity (Allfrey et al., 1964) and has been the most intensively studied histone modification. Acetylated histones are typically associated with transcriptionally active chromatin and deacetylated histones with inactive chromatin. In addition to the relationship between histone acetylation and the transcriptional capacity of chromatin, acetylation is also involved in processes such as replication and nucleosome assembly, higher-order chromatin packaging and the interactions of non-histone proteins with nucleosomes (Grant and Berger, 1999). In particular, the highly conserved H3 lysines at amino acid positions 9, 14, 18 and 23, and H4 lysines 5, 8, 12 and 16, are frequently targeted for acetylation (Roth et al., 2001) (Fig. 1.7). The neutralization of the histone tail basic charge by acetylation is thought to reduce the affinity for DNA and to alter histone:histone interactions between adjacent nucleosomes as well as the interactions of histones with other regulatory proteins (Grant and Berger, 1999; Roth et al., 2001). These changes contribute to a 'relaxed' chromatin environment believed to be permissive for transcription.

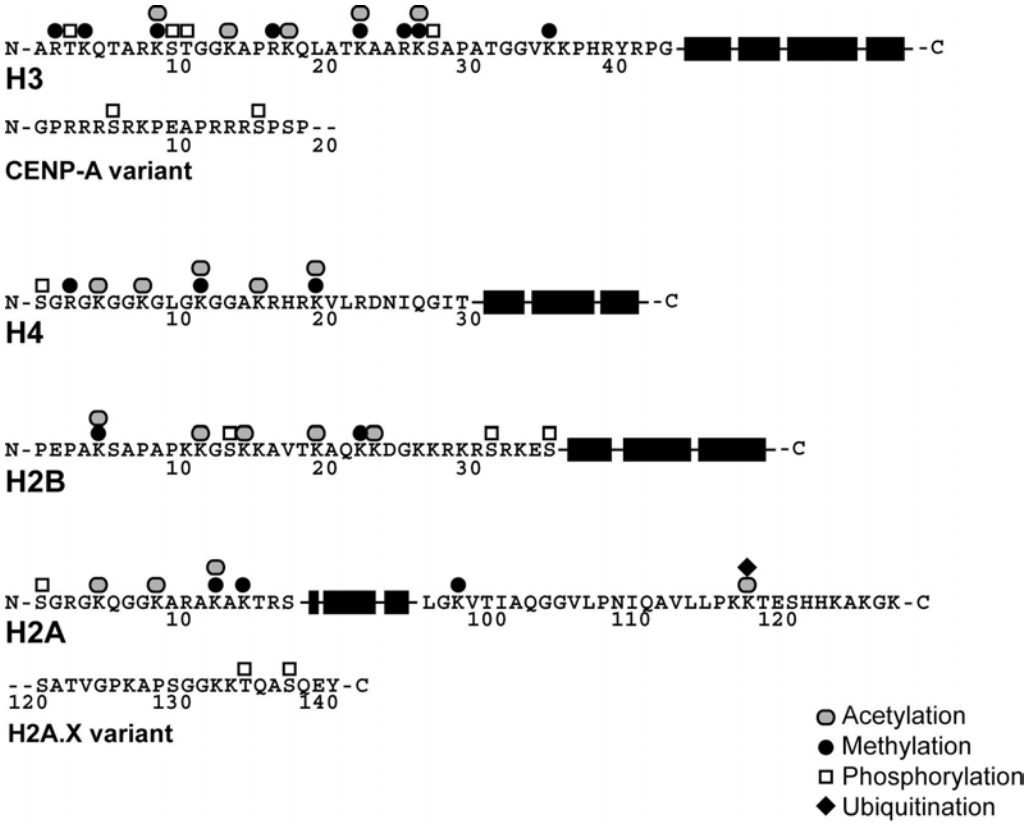

Figure 1.7 Known Post-Translational Histone Modifications. Illustrated are the amino acid sequences of the major areas of currently known post-translational modification in H3, the H3 amino-terminal (N) variant CENP-A, H4, H2B, H2A, and the H2A carboxy-terminal (C) variant H2A.X. As noted in the legend, common modifications include acetylation, methylation, phosphorylation and ubiquitination, and numbers represent amino acid position. Black boxes represent the alpha helices in the structured domains of the histone proteins. Adapted from Upstate Biotechnology, Inc. *'Histone Modification Map'*, 2004.

Molecular evidence for a direct link between acetylation and transcription was provided when the conserved transcriptional regulator GCN5 was found to harbor histone acetyltransferase (HAT) activity (Brownell et al., 1996). Since the identification of GCN5 as the archetypal nuclear HAT, numerous other coactivator proteins have been found to possess HAT activity and have frequently been identified as components of high molecular weight complexes composed of proteins with homology to transcriptional regulators (Grant and Berger, 1999). There are now several reported families of HATs which generate specific patterns of free and/or nucleosome associated histone acetylation. Importantly, many HATs have also been reported to modify other transcription factors, such as p53 (Gu and Roeder, 1997) and the basal transcription factors TFIIE and TFIIF (Imhof et al., 1997), suggesting a role for acetylation in gene and/or protein regulation beyond the modification of histones. This concept of HATs having non-histone protein substrates is becoming increasingly more popular and will undoubtedly be of great interest to many research areas of molecular biology.

The activity of HATs within a cell is balanced by the activity of the previously mentioned HDACs, which catalyze the removal of acetyl moieties from specific histone lysine residues, uncovering the lysine positive charge. The interaction between the positively charged lysines with DNA presumably restricts nucleosome mobility on the DNA, rendering a promoter region, for example, inaccessible to the transcription machinery (Ura et al., 1997). The HDAC superfamily is divided into three known classes: class I is similar to yeast RPD3, class II is similar to yeast HDAC1, and class III is similar to yeast Sir2. Class III

was discovered very recently and as a result, little is known regarding their expression, location, and specific activity (Thiagalingam et al., 2003). The numerous members of classes I and II, however, share a region of about 390 amino acids that contains the catalytic site and is conserved in eukaryotic cells. HDACs are targeted to histone tails by protein or protein complexes and, not surprisingly, both class I and II enzymes are found in the nucleus (Villar-Garea and Esteller, 2004).

An example of mislocalized HDAC activity in cancer is seen in the aforementioned AML associated t(8;21) translocation (Wang et al., 1999). This chromosomal abnormality fuses the DNA-binding domain of AML1, a transcriptional activator and repressor, with ETO, a nuclear phosphoprotein that is expressed in hematopoietic progenitors and which interacts with multiple HDACs, Sin3, and other co-repressors. The AML1/ETO fusion protein localizes to AML1 target genes, where it, in contrast to the wild-type AML1 protein, actively suppresses transcription via a co-repressor complex made up of N-CoR/Sin3/HDAC1. Since AML1 is required for differentiation of hematopoietic cells, AML1/ETO expression leads to a block in myeloid development and therefore assists leukemic transformation (Jones and Saha, 2002). Not surprisingly, since the detection of HDACs at inappropriately silenced tumor suppressor genes in human cancer, considerable effort has been put forward to identify inhibitors of this type of activity, which will be discussed in a subsequent section.

1.4.2.3. Histone Phosphorylation

In contrast to the relative wealth of information about the large number of HATs and HDACs, relatively little is known about the enzymes that generate other histone modifications. For example, specific histone kinases and phosphatases have largely remained elusive, yet progress has been made towards understanding the role of histone phosphorylation in processes such as transcription, DNA repair, apoptosis, and chromosome condensation (Cheung et al., 2000a). Although the mechanism by which phosphorylation contributes to transcriptional activation is not well understood, phosphorylation of H3 serine 10 (Fig. 1.7) has been reported to correlate with gene activation in mammalian cells (Thomson et al., 1999) and heat shock transcriptional induction in the *Drosophila melanogaster* fruit fly (Nowak and Corces, 2000). One possible mechanism behind this, analogous to acetylation, is that the addition of negatively charged phosphate groups to histone tails neutralizes the basic charge, reducing the affinity of histones for DNA. Furthermore, it has been reported that several acetyltransferases have increased HAT activity on H3 serine 10 phosphorylated substrates, and that mutation of serine 10 decreases activation of GCN5-regulated genes (Cheung et al., 2000b; Lo et al., 2000). Thus, it is possible that histone phosphorylation may contribute to transcriptional activation through the stimulation of HAT activity on the same histone tail.

Interestingly, phosphorylation of H3 at serine 10 has also been correlated with mitotic chromosome condensation, which is dephosphorylated at the completion of mitosis (Hendzel et al., 1997), and recent work suggests that the

kinase responsible for this modification is necessary for cells to pass through the G2/M transition (Van Hooser et al., 1998). Furthermore, as the induction of apoptosis also involves chromosome condensation, toxic agents have been shown to induce H3 serine 10 phosphorylation in association with apoptotic chromosome condensation (Waring et al., 1997). Serine 139 of the mammalian histone H2A variant, histone H2A.X (H2A.X) (Fig. 1.7), is also rapidly phosphorylated upon exposure to DNA damaging agents, which may play a role in facilitating end-joining repair of DNA (Rogakou et al., 1999). Recent work has also established a correlation between apoptosis and rapid phosphorylation of H2B serine 14 at sites of DNA double-strand breaks (Cheung et al., 2003; Fernandez-Capetillo et al., 2004). Although the functional roles that histone phosphorylation play in transcriptional activation and chromosome condensation are not fully actualized, the available data supports a role in the histone code hypothesis.

1.4.2.4. Histone Methylation

Methylation of histones was first described in 1964 (Murray, 1964), but the linkage of histone methylation and transcription was not evident until the H3 arginine specific HMT CARM1 was shown to interact and cooperate with the steroid hormone receptor coactivator GRIP-1 during transcriptional activation (Chen et al., 1999). Aside from arginine residues, multiple lysine residues residing within H3 (4, 9, and 27) and H4 (20) have been identified as substrates for preferential methylation (Fig. 1.7), and adding to the potential complexity, can be mono-, di-, or tri-methylated (Lachner and Jenuwein, 2002; Strahl et al., 1999).

The first lysine specific HMT to be discovered was the SUV39h1 protein, which has a catalytic site residing in a highly conserved SET domain which is directed against H3 lysine 9 (Rea et al., 2000). Since its identification, numerous other mammalian proteins have been characterized that contain SET domains, together comprising the SUV39, SET1, SET2, and RIZ families of lysine 9 HMTs (Kouzarides, 2002).

Mammalian SUV39h1 is a transcriptional repressor and localizes to transcriptionally silent heterochromatic sites, co-localizing with the transcriptional repressor heterochromatic protein 1 (HP1) (Aagaard et al., 1999). The HP1 protein is able to bind to H3 methylated at lysine 9 through interaction with its conserved chromodomain, yet how HP1 generates a highly compact chromatin structure is not known. It may link up several nucleosomes and/or may bring in other enzymatic activities necessary for compaction. Evidence that the SUV39h1/HP1 complex is involved in transcriptional repression at euchromatic loci comes from the finding that the RB protein recruits SUV39h1/HP1 to cell cycle controlling genes such as cyclin E (Nielsen et al., 2001). These results emphasize that histone lysine methylation and subsequent HP1 binding do not occur ubiquitously but rather are targeted events. Importantly, as H3 lysine 9 methylation can not take place if H3 lysine 9 is acetylated, the aforementioned HDAC activity of the RB repressor complex must first remove the acetyl moiety in order for lysine 9 methylation to proceed. This interaction has been documented in *Drosophila melanogaster* where specific HMT and HDAC proteins were identified in the same physical complex (Czermin et al., 2001), yet the exact mechanism behind HP1 in transcriptional

silencing at specific promoters, like that of its role at heterochromatic regions, is still unclear.

Other examples of genes potentially repressed by lysine methyltransferase activity are those regulated by IKAROS, a protein required for lymphoid development. In addition to recruiting HDAC activity, IKAROS also co-localizes with the HP1β isoform, suggesting that lysine 9 methyltransferase activity may be involved in its repressive mode of action (Brown et al., 1997).

Conversely, H3 lysine 4 methylation in mammalian cells and in yeast correlates with an active state of transcription (Strahl et al., 1999). Studies at the mating type loci in fission yeast have shown that there are large stretches of silent heterochromatin that contain histones methylated at H3 lysine 9 but that are devoid of H3 lysine 4 methylation. In contrast, the surrounding actively transcribed regions harbor H3 lysine 4 methylation but not that of H3 lysine 9. Importantly, removal of a boundary element in this model causes spreading of H3 lysine 9 methylation into the active domain resulting in depletion of H3 lysine 4 methylation (Noma et al., 2001). To add to the complexity, a recent yeast study using antibodies that distinguish between di- and tri-methylated H3 lysine 4 revealed that tri-methylation of H3 lysine 4 is specific for the active state of transcription, whereas di-methylated H3 lysine 4 exists in both active and repressed genes (Santos-Rosa et al., 2002). Thus, the number of methyl groups in a histone modification, and not just the targeted amino acid residue, appears to play a significant role in the functional consequences of histone methylation.

1.4.2.5. Histone Ubiquitination and ADP-ribosylation

A potentially vast number of other histone modifications within the unstructured amino-terminal tails and structured carboxyl-termini of histones may await discovery or warrant further investigation, including ubiquitination and ADP-ribosylation. Although very little is currently known about these histone modifications, early reports indicate that ubiquitination of H2B in yeast may play a role in mitosis and meiosis (Robzyk et al., 2000), and work in *Drosophila melanogaster* indicates potential roles for histone ubiquitination in transcriptional activation (Pham and Sauer, 2000).

ADP-ribosylation, which is the transfer of an ADP-ribose unit from nicotinamide adenine dinucleotide to a histone protein, has very recently been implicated in the enzymatic process involved in determining and/or maintaining the methylation pattern on genomic DNA (Zardo et al., 2003). Functional consequences of histone ADP-ribosylation, the enzymes responsible, and the specific histone substrates and amino acid targets are not well understood and are still very much in the early stages of exploration. However, the proposed relationship between histone ADP-ribosylation and DNA hypermethylation is very interesting and warrants further investigation. Other connections between histone modifications and DNA methylation have been established and will be further discussed in the subsequent section.

1.4.3. DNA Hypermethylation and Chromatin Remodeling

One of the major questions regarding DNA methylation is the identity of initial targeting mechanisms. In other words, how do patterns of DNA methylation originate? A seminal observation in the filamentous fungus *Neurospora crassa* has lead to the proposal that a specific chromatin structure involving methylated histones may be necessary for DNA methylation to take place (Tamaru and Selker, 2001). Specifically, the study showed that the *Neurospora crassa* gene dim-5, a histone H3 lysine 9 HMT, precedes and is a requirement for DNA methylation. This suggests a dependence on lysine 9 methylation for local DNA methylation to occur. Although dim-5 lacks a chromodomain, a model consistent with the SUV39h1/HP1 system suggests that H3 lysine 9 methylation could recruit a DNMT, either directly or through an intermediary factor, that has affinity for the H3 methylated lysine 9 position. Indeed, such a link has been described recently by the functional analysis of KRYPTONITE mutants in *Arabidopsis thaliana*. KRYPTONITE encodes a SET domain containing H3 lysine 9 specific HMT, which generates a substrate for a HP1-like protein that is known to interact with a DNMT, thereby recruiting DNA methylation activity to H3 lysine 9 methylated chromatin (Jackson et al., 2002). These results indicate an attractive hierarchy in epigenetic pathways where histone methylation functionally directs DNA methylation (Fig. 1.8).

However, a caveat of these two reports is that DNA methylation in fungi and plants is distinct from DNA methylation in mammalian cells. For example, Fuks et al. have shown that the mammalian MBD protein, MeCP2, in addition to

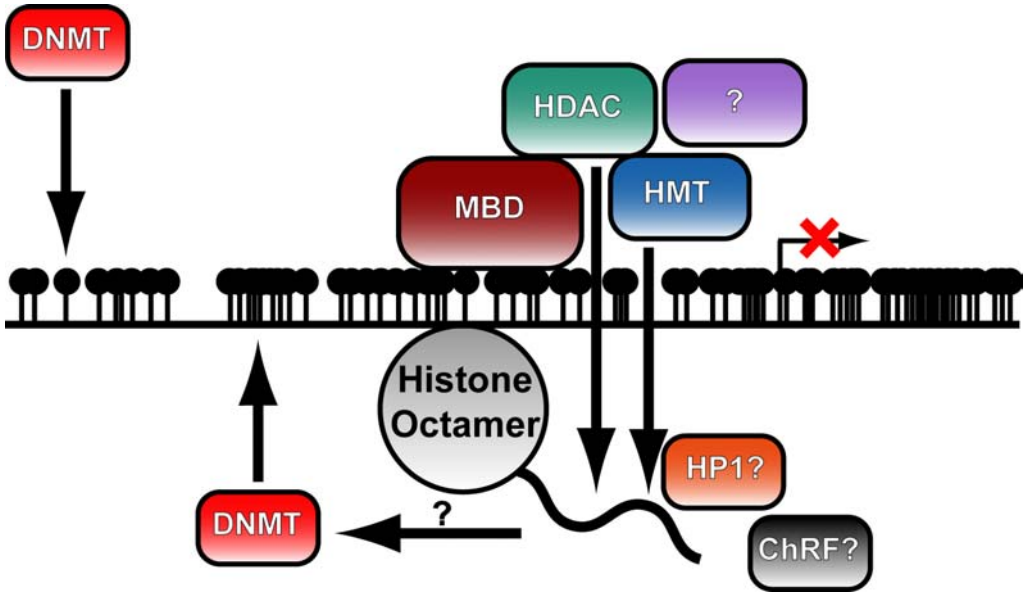

Figure 1.8 Model of DNA Hypermethylation Mediated Gene Silencing. The diagram illustrates a current model for epigenetic gene silencing involving promoter associated CpG island hypermethylation and post-translational modification of local histones. Black filled circles represent methyl-cytosines located within CpG dinucleotides (black vertical lines) and the bent arrow represents the major transcriptional start site. ChRF: chromatin remodeling factor.

interacting with a HDAC complex, associates with a HMT specific for H3 lysine 9 (Fuks et al., 2003b). This important study shows that the presence of MeCP2 on nucleosomes within the repressor region of the H19 gene coincides with an increase in H3 lysine 9 methylation and provides evidence for MeCP2 reinforcement of a repressive chromatin state by acting as a bridge between DNA methylation and histone methylation. Moreover, in a recent study involving the GSTP1 gene in prostate cancer, promoter hypermethylation was associated with the presence of MBD2 and local histone deacetylation (Stirzaker et al., 2004). The authors postulate a chronological model that includes promoter hypermethylation, MBD2 binding, H3 lysine 9 histone deacetylation, recruitment of more DNMTs and spreading of DNA hypermethylation, binding of MeCP2, and eventual H3 lysine 9 methylation. Again, it is an attractive hypothesis to envisage MBD proteins as co-repressors which target HDAC and HMT activities to hypermethylated promoters, (Fig. 1.8), yet the model fails to address the question of initial DNA methylation targeting mechanisms.

Although the exact targeting mechanism of aberrant epigenetic silencing in mammalian cells is as yet still unknown and the specific direction of modifications – either from DNA to histones or from histones to DNA – is still uncertain, it is clear that a strong correlation exists between DNA hypermethylation and histone deacetylation. Interestingly, between DNA hypermethylation and histone deacetylation, DNA hypermethylation appears to play a dominant role in the silencing of genes in cancer cells. Evidence for this comes from the fact that pharmacological inhibition of HDAC fails to induce transcription of

hypermethylated genes (Cameron et al., 1999a; Suzuki et al., 2002). In contrast, genes silenced by promoter hypermethylation are routinely reactivated when exposed to pharmacological inhibition or genetic knock out of DNMT activity. Moreover, synergistic reactivation of hypermethylated genes occurs *in vitro* when cells are treated with a combination of demethylating drugs and HDAC inhibitors, reinforcing the hypothesis that DNA methylation is involved in transcription modulation by 'locking' in a repressive chromatin conformation.

1.4.4. Inhibitors of DNA Methylation and Histone Deacetylation

The fact that many human diseases, including cancer, have a significant epigenetic etiology has encouraged the development of novel therapeutic options. Many agents have been discovered that alter methylation patterns on DNA or the modification of histones, and several of these agents are currently being evaluated in clinical trials.

1.4.4.1. DNA Methyltransferase Inhibition

Inhibitors of DNA methylation rapidly reactivate the expression of genes that have undergone promoter methylation dependent gene silencing, particularly if this silencing has occurred in a pathological situation. The first described inhibitors of methylation, 5-azacytidine (5-Aza) and its deoxy analog 5-Aza-dC, were initially developed as chemotherapeutic cytotoxic agents (Sorm et al., 1964) (Fig. 1.9), but it was subsequently discovered that they were also powerful inhibitors of DNA methylation and could induce gene expression and differentiation in cultured cells

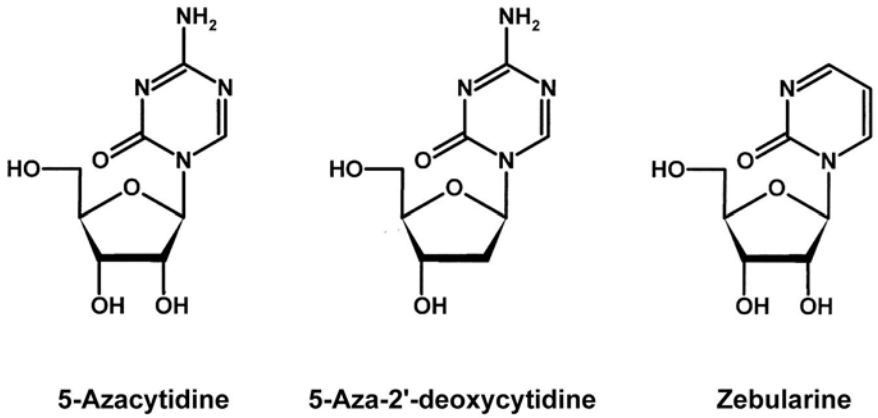

Figure 1.9 Chemical Structures of Common DNMT Inhibitors. Illustrated are the chemical structures and names of the common cytosine analog inhibitors of DNA methylation. Structures were constructed using ChemSketch Version 5.12 software (Advanced Chemistry Development Inc., Toronto, ON, Canada).

(Constantinides et al., 1977; Jones and Taylor, 1980). Once introduced into a nucleus, both nucleoside analogues are converted to the deoxynucleotide triphosphates and are then incorporated into replicating DNA in place of cytosine. As such, they are primarily active in S phase cells where they serve as powerful inhibitors of DNA methylation. DNMTs get trapped on DNA containing modified bases such as azacytosine, 5-fluorocytosine, pseudoisocytosine and zebularine, resulting in the formation of heritable demethylated DNA (Jones and Taylor, 1980; Zhou et al., 2002b). Of note, the covalent attachment of various DNMTs to DNA may well be responsible for the cytotoxicities of these agents, particularly at high doses (Michalowsky and Jones, 1987).

A disadvantage of the aza-nucleosides is their instability in aqueous solutions, yet this may be overcome by the use of other analogues such as zebularine or 5-fluoro-2'deoxycytidine, which also inhibit DNA methylation following incorporation into DNA. For example, in a recent study involving tumor burdened mice, oral administration of zebularine, owing to its stability, was effective at demethylating the CDKI p16 *in vivo* in association with reducing tumor volume (Cheng et al., 2003). Non-cytosine analogs such as procaine and the related compound procainamide, which are normally used to treat cardiac arrhythmias, are other examples of inhibitors of DNA methylation (Villar-Garea et al., 2003). In addition, natural products derived from tea and sponges have shown DNA demethylating activity *in vitro* (Fang et al., 2003; Pina et al., 2003). Clinical trials with antisense oligonucleotides that target the DNMT enzymes are also underway (Yan et al., 2003).

As mentioned, inhibitors of epigenetic silencing are currently being evaluated in clinical trials, the most promising being 5-Aza and 5-Aza-dC in the treatment of hematological malignancies. In a relatively large phase II study of elderly patients with intermediate or high-risk MDS, Wijermans et al. found low dose 5-Aza-dC to be well tolerated with the only major side effect being myelosuppression (Wijermans et al., 2000). Within the treated patient population, 20% achieved a complete remission that was associated with cytogenetic remission and another 28% showed significant clinical improvement (Lubbert et al., 2001). A phase II study of 5-Aza-dC in CML, conducted prior to the approval of imatinib mesylate, also provided promising data (Kantarjian et al., 2003). In this report complete hematologic responses were observed in blastic, accelerated, and chronic phase patients, and similar to the MDS study, the main side effect was myelosuppression. Furthermore, in a very recent phase I study, Issa et al. evaluated low-dose prolonged exposure schedules of 5-Aza-dC in relapsed/refractory leukemias (Issa et al., 2004). The drug was well tolerated in all patients, including those with AML/MDS, CML and ALL, and responses were seen at all dose levels. However, the authors report that fewer responses were seen when the dose was escalated or prolonged and concluded that although 5-Aza-dC is effective in myeloid malignancies, low doses are as or more effective than higher doses.

With increasing evidence of pre-clinical efficacy, the possibility of optimally combining 5-Aza-dC, or other DNMT inhibitors, with other drugs is inevitably being addressed. Although DNMT and HDAC inhibitors act synergistically with respect to gene reactivation and growth inhibition *in vitro*,

clinical trials of such combinations have not yet been reported. Furthermore, combination treatment including 5-Aza-dC and conventional chemotherapeutics, although still in development, may be effective in the treatment of various tumor types. For example, 5-Aza-dC has recently been shown to act synergistically *in vitro* with imatinib mesylate in the treatment of resistant CML cell lines (La Rosee et al., 2004), underscoring the importance of developing novel therapy combinations and the difficulty in treating drug resistant tumors.

1.4.4.2. Histone Deacetylase Inhibition

The biochemical structures of HDAC inhibitors are extremely heterogeneous, from simple structures, such as valproate, to more elaborate designs, such as the benzamide MS-275. The very potent hydroxamic acid family, which includes one of the first HDAC inhibitors to be described, TSA (Fig. 1.10) (Yoshida et al., 1990), is the broadest class of HDAC inhibitor. Among many other drugs developed in this class, one of the more common is suberoylanilide hydroxamic acid (SAHA) (Fig. 1.10). Presently involved in phase I/II clinical trials, SAHA has recently been shown to induce the CDKI p21, like many other HDACs, in the human myeloma cell line ARP-1 (Gui et al., 2004). This response was associated with increased histone acetylation, release of HDAC1, and increased DNase I sensitivity at the p21 promoter identifying, in part, a selective drug effect that supports cell growth inhibition. Additional classes of HDAC inhibitors exist including, among others, short chain carboxylic acids, benzamides, and epoxides.

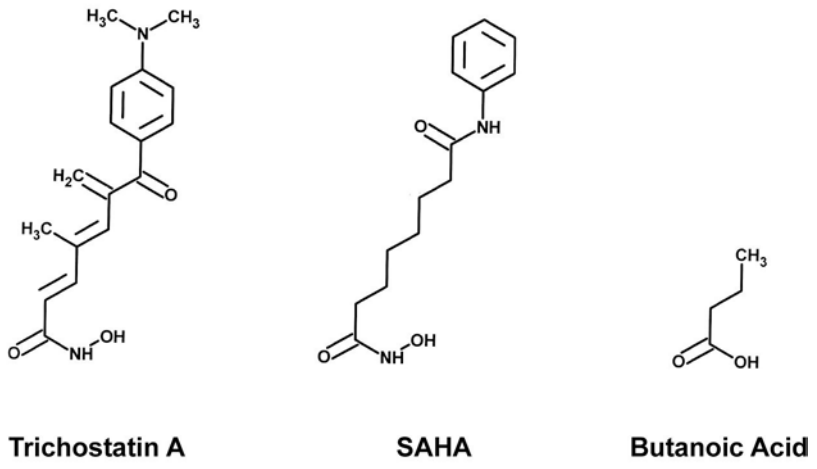

Figure 1.10 Chemical Structures of Common HDAC Inhibitors. Illustrated are the chemical structures and names of some common HDAC inhibitors. TSA and SAHA are hydroxamic acids and Butanoic Acid is a short chain fatty acid. Structures were constructed using ChemSketch Version 5.12 software (Advanced Chemistry Development Inc., Toronto, ON, Canada).

The anticancer effects of HDAC inhibitors are believed to be mediated by reactivation of tumor suppressor gene expression in malignant cells. However, many other secondary factors may also play an important indirect role by mediating differentiation, cell cycle arrest, and apoptosis (Villar-Garea and Esteller, 2004). As mentioned, treatment of cancer cell lines with HDAC inhibitors leads to an increase in global acetylated H3 and H4, which is generally associated with gene expression, however, several studies have revealed that the number of significantly repressed genes is similar to the number of upregulated transcripts (Glaser et al., 2003), underscoring the complex relationship between chromatin structure, histone modification, and transcription. Although most HDAC inhibitors act by similar mechanisms, blocking access of acetyl-lysine residues to the highly conserved HDAC catalytic pocket, these drugs have markedly different effects on the resulting gene expression patterns, even when treated on the same cell line (Glaser et al., 2003). This behavior may be due in part to differences in drug specificity for the various HDACs.

As previously mentioned, HDAC inhibitors act synergistically *in vitro* with demethylating agents, which was initially observed in colon cancer cell lines at hypermethylated tumor suppressor gene loci including the CDKI p16 (Cameron et al., 1999a), but have yet to be established together in published clinical trials. However, a host of HDAC inhibitors are currently undergoing phase I and II evaluation as monotherapy as well as in combination with other cytotoxics and differentiation agents (Kelly et al., 2002b). For example, phenylbutyrate, a short chain fatty acid HDAC inhibitor (Fig. 1.10), has recently exhibited clinical

effectiveness with continuous infusion in both myeloid malignancies and solid tumors with the most common side effects being dyspepsia, fatigue, and nausea (Gilbert et al., 2001; Gore et al., 2002).

This agent has also been examined in the M3 AML subtype APL, which accounts for approximately 10% of all AML patients (Table 1.1). As mentioned, APL is characterized by the t(15;17) chromosomal translocation that involves the RARα gene on chromosome 17 and the exquisite sensitivity of APL blasts to the differentiating action of ATRA (Melnick and Licht, 1999). ATRA accomplishes its therapeutic effect by triggering terminal differentiation of APL blasts into mature granulocytes, yet treatment often induces transient disease remission with inevitable relapse if remission is not consolidated with chemotherapy. Based on the *in vitro* data which illustrates that the oncoprotein encoded by the fusion gene in APL suppresses gene transcription by the recruitment of a HDAC, APL patients refractory to ATRA have been treated with a combination of phenylbutyrate and ATRA (Warrell et al., 1998; Zhou et al., 2002a). Phenylbutyrate induced accumulation of acetylated histones in the acute promyelocytic cells and restored ATRA sensitivity resulting in transient clinical improvement in 20% of patients (Zhou et al., 2002a). Of the two phase I clinical trials using phenylbutyrate that have now been reported (Carducci et al., 2001; Gilbert et al., 2001), a biologically active dose was attained with minimal clinical and metabolic side effects and, although no partial or complete remissions were induced, one trial reported that 25% of patients achieved disease stabilization for more than six months while on the drug (Gilbert et al., 2001).

Owing to the early success of HDAC inhibitors as anticancer agents, phase I and II studies utilizing phenylbutyrate and ATRA, phenylbutyrate and azacytidine, phenylbutyrate, dexamethasone and GM-CSF, plus other combinations incorporating other HDAC inhibitors are currently ongoing in a spectrum of human malignancies (Johnstone, 2002). These drugs appear to be well tolerated at doses required to acetylate histones and achieve clinical outcomes, and their use in combination therapies is an exciting area that will likely be persued in the clinic. Taken together, these exciting *in vitro* and *in vivo* data illustrate the clinical potential of HDAC inhibitors for the treatment of leukemias and solid tumors.

2. HYPOTHESIS AND SPECIFIC AIMS

RATIONALE: Promoter hypermethylation mediated gene silencing is a frequent finding in many different types of human cancer (Esteller et al., 2001; Paz et al., 2003). The genes affected by epigenetic silencing are known to have important roles in cellular processes including tumor suppression, cell cycle regulation, apoptosis, DNA repair, and metastastic potential. Furthermore, sites of altered DNA methylation constitute some of the most promising molecular markers for use in early cancer diagnosis, predicting cancer risk status, and monitoring prognosis. Understanding the mechanisms by which hypermethylation effects gene silencing should allow the design of future therapies that aim to improve clinical outcomes by restoring the expression of genes critical to cellular behavior. It is also important to develop strategies to determine the spectrum of genes silenced by hypermethylation in specific cancer types in order to better understand the molecular pathogenesis of these diseases and develop improved diagnostic markers. The work of several laboratories, including that of Dr. DeCoteau's, have now identified a role for promoter hypermethylation in the pathogenesis of human leukemia and lymphoma (Scott et al., 2003; Scott et al., 2004; Toyota et al., 2001). The recent resurgence in the clinical use of pharmacological agents capable of reversing promoter methylation in the treatment of myeloid malignancies

underscores the importance of epigenetics to malignant myeloid biology. The availability of these agents also provides opportunities to manipulate relevant *in vitro* models to gain insight into the mechanisms by which hypermethylation mediates gene silencing and to identify the spectrum of genes silenced by this mechanism in these aggressively growing hematopoietic cancers. Taken together, these observations constitute the basis for both the general hypotheses and specific aims of this thesis, which are detailed below.

HYPOTHESES: In acute myeloid leukemia (AML), aberrantly methylated promoter alleles of tumor suppressor genes are associated with histone modifications that confer a transcriptionally repressive chromatin conformation. For some tumor suppressor genes, pharmacological inhibition of DNA methylation leads to a reversal of transcriptionally repressive histone modifications and the restoration of gene expression.

SPECIFIC AIM ONE: To investigate the relationship between aberrant promoter hypermethylation, histone modifications, and tumor suppressor gene silencing in a cell line model of relevance to the *in vivo* pathogenesis of AML. (Experiments pertaining to this AIM are outlined in Sections 4.1 and 4.2.)

SPECIFIC AIM TWO: To determine if zebularine, a novel DNA methylation inhibitor, can restore expression of a tumor suppressor gene by a mechanism

involving promoter demethylation and histone modification in a cell line model of AML. (Experiments pertaining to this AIM are outlined in Section 4.3.)

SPECIFIC AIM THREE: To use pharmacological inhibition of DNA methylation to discover novel genes silenced by promoter hypermethylation in AML. (Experiments pertaining to this AIM are outlined in Section 4.4.)

3. MATERIALS AND METHODS

3.1 Reagents and Suppliers

The reagents used for experiments in this thesis were all molecular biology or reagent grade and are listed in Table 3.1. Several of the procedures used in this study were performed using commercially available kits, which are listed in Table 3.2. Table 3.3 lists the companies from which all reagents and kits were obtained.

Table 3.1 Reagents and Suppliers Used in This Study

Reagent	Supplier Name
[^{3}H]-thymidine (42 Ci/mmol; 1.55 TBq/mmol)	Amersham Biotech
[α^{32}P]-dCTP (800 Ci/mmol; 29.6 TBq/mmol)	Perkin Elmer
[α^{32}P]-UTP (3000 Ci/mmol; 111 TBq/mmol)	Amersham Biotech
β-mercaptoethanol	Sigma
5-aza-2'-deoxycytidine	Sigma
acetic acid	BDH
agarose	Invitrogen Life Technologies
ammonium acetate	EMD Chemicals
ampicillin	Fisher
aprotinin	Sigma
APS	Invitrogen Life Technologies
bacto-agar	Invitrogen Life Technologies
bacto-tryptone	Difco Laboratories
bacto-yeast extract	Difco Laboratories
borate	BDH
Bradford protein reagent	Bio-Rad
bromophenol blue	BDH
calf thymus DNA	Sigma
Coomassie Brilliant Blue R-250	Bio-Rad
CpGenome universally methylated DNA	Chemicon
Cyanine 3-dCTP	NEN Life Science

Cyanine 5-dCTP	NEN Life Science
dATP	Fermentas
dCTP	Fermentas
dGTP	Fermentas
DIG Easy Hyb solution	Roche
dNTP Mix	Fermentas
DTT	Invitrogen Life Technologies
dTTP	Fermentas
EDTA	EMD Chemicals
ethidium bromide	Invitrogen Life Technologies
fetal bovine serum	HyClone
Ficoll-Paque PLUS	Amersham Biotech
formaldehyde	BDH
formamide	BDH
GeneRuler 100 bp DNA ladder	Fermentas
glycerol	Invitrogen Life Technologies
glycine	EMD Chemicals
GlycoBlue coprecipitant	Ambion
HCl	EMD Chemicals
HEPES	BDH
HotStar*Taq* Polymerase	Qiagen
Hybond-N+ nylon membrane	Amersham Biotech
Hybri-slips	Sigma
hydroquinone	BDH
Hyperfilm MP	Amersham Biotech
IMDM	Invitrogen Life Technologies
isopropanol	EMD Chemicals
KCl	Sigma
magnesium acetate	Sigma
methanol	BDH
$MgCl_2$	BDH
MOPS	Sigma
MTT	Sigma
N,N-methylene-bis-acrylamide	Invitrogen Life Technologies
NaCl	BDH
NaOH	BDH
non-fat dry milk	Carnation
NP-40	Sigma
nuclease-free water	Ambion
penicillin/streptomycin 100X mix	Invitrogen Life Technologies
pepstatin A	Sigma
phenol:chloroform:isoamyl alcohol (25:24:1)	Ambion
PMSF	Sigma
polyacrylamide	Invitrogen Life Technologies
polyethylene glycol-8000	EMD Chemicals
prestained protein molecular weight markers	Fermentas

propidium iodide	Sigma
proteinase K	Qiagen
REDTaq DNA Polymerase	Sigma
rhGM-CSF	R&D Systems
rhIL-3	R&D Systems
RNase	Worthington
RNasin	Promega
RPMI-1640	Invitrogen Life Technologies
rTdT	Invitrogen Life Technologies
SDS	BDH
sodium acetate	BDH
sodium azide	Sigma
sodium bicarbonate	BDH
sodium bisulfite	Sigma
sodium citrate	BDH
sodium deoxycholate	Sigma
sodium fluoride	BDH
sodium orthovanadate	Sigma
sodium pyruvate	Sigma
TEMED	EMD Chemicals
Trans-Blot nitrocellulose	Bio-Rad
trichostatin A	Sigma
Tris	EMD Chemicals
Triton X-100	Sigma
TRIzol	Invitrogen Life Technologies
Trypan Blue	Invitrogen Life Technologies
urea	EMD Chemicals
yeast tRNA	Invitrogen Life Technologies
zebularine	Calbiochem

Table 3.2 Commercially Available Kits Used in This Study

Commercially Used Kits	**Company**
Chromatin Immunoprecipitation Assay kit	Upstate Biotechnology
Immun-Star HRP Chemiluminescent kit	Bio-Rad
In Vitro Transcription kit	BD Biosciences
M-MLV RT system	Promega
QIAprep Miniprep kit	Qiagen
QIAquick Gel Extraction kit	Qiagen
QIAquick PCR Purification kit	Qiagen
RNase-Free DNase set	Qiagen
RNeasy Mini kit	Qiagen
RPA kit	BD Biosciences

Superscript RT system	Invitrogen Life Technologies
TOPO TA Cloning kit	Invitrogen Life Technologies
Wizard DNA Clean-up kit	Promega

Table 3.3 Names and Addresses of Suppliers

Supplier	Address
Abcam	Abcam Ltd., Cambridge, UK
Ambion	Ambion, Inc., Austin, Texas, USA
Amersham Biotech	Amersham Biotech, Inc., Baie d'Urfe, Canada
BD Biosciences	BD Biosciences, Mississauga, Canada
BDH	BDH Inc., Toronto, Canada
Bio-Rad	Bio-Rad Laboratories, Ltd., Mississauga, Canada
Calbiochem	EMD Biosciences, La Jolla, CA, USA
Chemicon	Serologicals Corp., Norcross, GA, USA
Difco Laboratories	Difco Laboratories, Detroit, MI, USA
DSMZ	DSMZ, Braunschweig, Germany
EMD Chemicals	EMD Biosciences, La Jolla, CA, USA
Fermentas	Fermentas Canada, Burlington, Canada
Fisher	Fisher Scientific Ltd., Nepean, Canada
HyClone	HyClone Laboratories, Logan, UT, USA
Invitrogen Life Technologies	Invitrogen Life Technologies, Burlington, Canada
Millipore	Millipore Ltd., Nepean, Canada
NEN Life Science	DuPont NEN Research Products, Boston, MA, USA
Perkin Elmer	Perkin Elmer Inc., Wellesley, MA, USA
Promega	Promega Corporation, Madison, WI, USA
Qiagen	Qiagen, Mississauga, Ontario, Canada
R&D Systems	R&D Systems Inc., Minneapolis, MN, USA
Roche Diagnostics	Roche, Indianapolis, IN, USA
Santa Cruz Biotechnology	Santa Cruz Biotechnology, Santa Cruz, CA, USA
Sigma	Sigma-Aldrich, St. Louis, MO, USA
Upstate Biotechnology	Upstate Biotechnology, Lake Placid, NY, USA
Worthington Biochemical	Worthington Biochemical Corp., Lakewood, NJ, USA

3.2 Oligonucleotides

Table 3.4 lists all the primers and probes used for experiments in this study with their optimal annealing or hybridization temperatures listed. All oligonucleotides were purchased from Invitrogen.

Table 3.4 Sequences and Optimal Annealing/Hybridization Temperatures of Primers and Probes Used in This Study

Name[a]	Sequence[b]	Temp.
p15-RTPCR-F	5'-ATGCGCGAGGAGAACAAGGG-3'	63.8ºC
p15-RTPCR-R	5'-GTACCCTGCAACGTCGCGGT-3'	
Actin-RTPCR-F	5'-CCAAGGCCAACCGCGAGAAGAT-3'	61.3ºC
Actin-RTPCR-R	5'-TTGCTCGAAGTCCAGGGCGA-3'	
p15-MSP-U-F	5'-**T**G**T**GA**T**G**T**G**TTT**G**T**A**TTT**TG**T**GG**TT**-3'	60.0ºC
p15-MSP-U-R	5'-CC**A**T**A**CA**AT**AACC**A**AA**A**C**AA**CC**AA**-3'	
p15-MSP-M-F	5'-GCGT**T**CG**T**A**TTT**TGCGG**TT**-3'	60.0ºC
p15-MSP-M-R	5'-CGT**A**CA**AT**AACCGA**A**CG**A**CCG**A**-3'	
p15-TTGE-F	5'-TGG**TT**T**TTT**A**TT**T**TG**TT**AGA-3'	52.0ºC
p15-TTGE-R	5'-[gcgcgcgcgcgc]-ACACTCTTCCCTTCTTTCCC-3'	
p15-TTGE-probe	5'-AGGGTAATGAAG**T**TGAG**TTT**-3'	40.5ºC
p21-MSP-U-F	5'-GG**T**GG**T**G**T**GGTGGG**TT**GAG**T**-3'	62.0ºC
p21-MSP-U-R	5'-**A**C**A**AATCC**A**C**A**CCCA**A**CTCC-3'	
p21-MSP-M-F	5'-**TTT**CGGGGAGGGCGGT**TT**CGGGCGGCGCGG-3'	67.0ºC
p21-MSP-M-R	5'-CG**A**T**A**CCTCG**A**CGAATCCGC-3'	
p21-TTGE-F	5'-AAAG**TT**AGATTTGTGG**T**T**T**A**T**T-3'	51.0ºC
p21-TTGE-R	5'-[gcgcgcgcgcgcgcgcgcgc]-CTCTCACCTCCTCT**A**A**A**T**A**CCT-3'	
p21-TTGE-probe	5'-GGG**T**AG**TT**AGGAG**TT**TGGG**T**-3'	44.0ºC
p27-MSP-U-F	5'-GAGA**T**T**T**GG**T**GG**TT**GGG**TT**G-3'	60.0ºC

p27-MSP-U-R	5'-**A**CAC**A**ACC**A**CCTCTCTC**A**C**A**-3'	
p27-MSP-M-F	5'-T**T**AGCGGAGA**T**TCGGCGG**T**C-3'	58.0ºC
p27-MSP-M-R	5'-CG**AA**TCT**A**CACGACCGCCTC-3'	
p27-TTGE-F	5'-GGGT**T**TGTG**T**TTTTTGG**TT**-3'	52.5ºC
p27-TTGE-R	5'-[gcgcgcgcgcgcgcgc]-T**A**CA**A**TACTTCTCCAA**A**TCC-3'	
p27-TTGE-probe	5'-GGGAG**TTT**TAG**TT**TGGAG-3'	39.0ºC
p15-ChIP-F	5'-GCAGGCTTCCCCGCCCTCGTGACGC-3'	64.6ºC
p15-ChIP-R	5'-ATTACCCTCCCGTCGTCCTTCTGC-3'	
MT1H-RTPCR-F	5'-AAATGCACCTCCTGCAAGAAGAGCTGCTG-3'	62.5ºC
MT1H-RTPCR-R	5'-AGGAATGTAGCAAATGAGTCGGAGTTGTAG-3'	
MT1G-RTPCR-F	5'-ACCCACTGCCTCTTCCCTT-3'	57.0ºC
MT1G-RTPCR-R	5'-TTGTACTTGGGAGCAGGG-3'	
SAT-RTPCR-F	5'-CACCTCCTCCTACTGTTC-3'	52.0ºC
SAT-RTPCR-R	5'-GATCAGAAGCACCTCTTC-3'	
MT1E-RTPCR-F	5'-CTCCTGCAAGTGCAAAGAGTGCAA-3'	55.0ºC
MT1E-RTPCR-R	5'-GGCACAGCTCTGTTCTGAAACCAT-3'	
IFI30-RTPCR-F	5'-CCTGGTTAAAGGCGCTTA-3'	57.0ºC
IFI30-RTPCR-R	5'-TGGTCAGGAAGGCTAGCT-3'	
GPX4-RTPCR-F	5'-CCTTTGCCGCCTACAGCC-3'	57.0ºC
GPX4-RTPCR-R	5'-TCTCTATCACCAGGGGCT-3'	
MT2A-RTPCR-F	5'-CGTGCAACCTGTCCCGACTCTA-3'	58.6ºC
MT2A-RTPCR-R	5'-AACGGTCACGGTCAGGGTT-3'	
MT1H-MSP-U-F	5'-T**T**T**T**GGGG**T**A**T**G**T**GGAA**T**G**TT**A-3'	52.0ºC
MT1H-MSP-U-R	5'-CACT**AA****A**TCC**A**AA**A**CC**A**CCC**A**C**A**-3'	
MT1H-MSP-M-F	5'-T**T**TCGGGG**T**ACGCGGAACG**TT**A-3'	57.0ºC
MT1H-MSP-M-R	5'-CT**AA****A**TCCGA**A**CCGCCCGCG**A**T-3'	
MT1G-MSP-U-F	5'-**TT****T**G**T**GAG**TT**GGTG**T**GAAAGGG-3'	52.0ºC
MT1G-MSP-U-R	5'-**AA**CC**AAA**C**A**CA**A**AC**A**CCCC**A**CC-3'	
MT1G-MSP-M-F	5'-GAG**T**CGGTGCGAAAGGGG**T**CGT**TT**-3'	57.0ºC

MT1G-MSP-M-R	5'-**A**ACG**AAA**CCG**AA**CGCA**A**ACGCC-3'	
SAT-MSP-U-F	5'-GAGGT**TTT**A**TT**TTA**T**GG**TTT**GAGG**T**-3'	51.0ºC
SAT-MSP-U-R	5'-**A**AA**T**A**AA**CATAAACACCA**A**TC**AA**C-3'	
SAT-MSP-M-F	5'-GT**TTT**A**TT**TTACGG**TT**CGAGGC-3'	55.0ºC
SAT-MSP-M-R	5'-GA**A**TA**A**ACG**A**ATAAACACCA**A**TCG**A**-3'	
MT1E-MSP-U-F	5'-GGGTGGAGA**T**G**TTT**G**T**GA**T**G**TT**-3'	52.0ºC
MT1E-MSP-U-R	5'-CC**A**CA**A**AA**AAA**CC**AAA**C**A**CACC-3'	
MT1E-MSP-M-F	5'-G**TT**CGCGACG**TT**AAGG**T**TGGGGT**T**-3'	57.0ºC
MT1E-MSP-M-R	5'-ACG**AA**A**A**TCG**AA**CCG**AA**CGCA**A**-3'	
IFI30-MSP-U-F	5'-**T**GTAT**TTT**GTAT**T**TGAGAGG**T**G**T**-3'	55.0ºC
IFI30-MSP-U-R	5'-TC**A**CT**AA**A**A**C**AA**TA**AA**CAA**A**TC**A**CT-3'	
IFI30-MSP-M-F	5'-TTCG**T**AT**TT**CG**T**AT**T**TGAGAGGC-3'	55.0ºC
IFI30-MSP-M-R	5'-GCT**AA**A**A**CG**A**TA**AA**CAA**A**TCGCT-3'	
GPX4-MSP-U-F	5'-AGA**T**A**T**AAG**T**GAG**T**ATG**T**G**T**AGT**T**G**T**-3'	55.0ºC
GPX4-MSP-U-R	5'-TCATT**AA**TCA**A**AC**A**C**A**TC**AA**TA**T**TA**AA**C**A**C-3'	
GPX4-MSP-M-F	5'-TAGA**T**A**T**AAGCGAG**T**ATGCG**T**AGTC-3'	55.0ºC
GPX4-MSP-M-R	5'-A**A**ACGCGTCG**A**T**A**TT**AA**A**A**CG-3'	
MT2A-MSP-U-F	5'-G**T**G**T**G**T**TAA**T**GG**TTT**AGGTT**T**G-3'	50.0ºC
MT2A -MSP-U-R	5'-CC**A**C**A**CT**AA**TCACTTA**C**A**A**CT-3'	
MT2A -MSP-M-F	5'-**TT**CGCGCG**T**TAACGG**TT**T**A**GGT-3'	57.0ºC
MT2A -MSP-M-R	5'-GCACT**AAA**CCG**AA**CGCACCCG**A**-3'	
MT1H-ChIP-F	5'-CCAAAGGGCGGGAGTAGCAGGTAA-3'	57.0ºC
MT1H-ChIP-R	5'-CGCAACAGTTGGCAGCTCCTTT-3'	

[a] F: forward (sense) primers; R: reverse (antisense) primers; U: MSP primers specific for bisulfite modified, unmethylated DNA; M: MSP primers specific for bisulfite modified, methylated DNA.
[b] MSP primer sets were designed to distinguish methylated from unmethylated DNA following bisulfite modification. Sequence differences between primers and unmodified DNA are in boldface type and potential sites of sense strand cytosine methylation within CpG dinucleotides are underlined.

3.3 Antibodies

Table 3.5 lists all the primary and secondary antibodies used for experiments in this study and the supplier from which they were purchased.

Table 3.5 Antibodies Used in This Study

Antibody	Supplier
anti-Actin	Santa Cruz
anti-HDAC1	Upstate Biotechnology
anti-histone H2A.X-phospho-S139	Upstate Biotechnology
anti-histone H2B	Upstate Biotechnology
anti-histone H2B-phospho-S14	Upstate Biotechnology
anti-histone H3	Upstate Biotechnology
anti-histone H3-acetyl-K9/K14	Upstate Biotechnology
anti-histone H3-acetyl-K18	Upstate Biotechnology
anti-histone H3-acetyl-K23	Upstate Biotechnology
anti-histone H3-dimethyl-K4	Upstate Biotechnology
anti-histone H3-dimethyl-K9	Upstate Biotechnology
anti-histone H3-dimethyl-R17	Upstate Biotechnology
anti-histone H3-phospho-S10	Upstate Biotechnology
anti-histone H4-acetyl-K5/8/12/16	Upstate Biotechnology
anti-MeCP2	Upstate Biotechnology
anti-SUV39h1	Upstate Biotechnology
HRP conjugated Bovine anti-Goat IgG	Santa Cruz
HRP conjugated Goat anti-Mouse IgG	Bio-Rad
HRP conjugated Goat anti-Rabbit IgG	Bio-Rad

3.4. Patient Specimens

AML patient and control samples were obtained from an established bank of marrow aspirate and peripheral blood specimens at the University of Saskatchewan. Sample collection, banking and study were performed according to guidelines approved by the University of Saskatchewan ethics review board. DNA and RNA were extracted from banked samples of marrow aspirate (n = 23) or

peripheral blood (n = 17) obtained from 21 adult AML patients (ages 22 - 86) at diagnosis (n = 36) or first relapse (n = 4). Banked samples were prepared by isolating cells from fresh bone marrow aspirate or peripheral blood specimens by Ficoll Paque (Amersham Biosciences) density centrifugation, followed by cryopreservation in DMSO. All samples were required to fulfill the following criteria for banking: i) the blast cell population accounted for >75% of all nucleated cells in the aspirate or peripheral blood sample prior to Ficoll Paque density centrifugation and ii) the sample contained at least 5 x 10^7 nucleated cells with a cellular viability >90% (as assessed by Trypan Blue dye exclusion) following Ficoll Paque separation. The patients included the following FAB subtypes: M0 (n = 3), M1 (n = 10), M2 (n = 13), M4 (n = 10), M5a (n = 1), M5b (n = 1) and M6 (n = 2) as assessed by light microscopy, cytochemical stains and multiparameter flow cytometry at diagnosis. Control DNA samples were randomly selected from a bank maintained at the University of Saskatchewan, Saskatoon, SK, Canada. The source of these DNA samples was peripheral blood mononuclear cells from adults who had been investigated for Factor V Leiden and found to be negative (n = 13).

3.5. Cell Lines and Tissue Culture

3.5.1. Cell Lines and Standard Culture Conditions

Human AML cell lines HL60, KG1, KG1A, AML193, THP1, HEL and K562 were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ) and are characterized in Table 3.6. HL60, KG1, KG1A and AML193 were cultured in IMDM media and 20% (v/v) fetal bovine serum (FBS,

HyClone); AML193 culture was supplemented with 2 ng/ml GM-CSF and 3 units/ml IL-3 (R&D Systems). THP1, HEL and K562 were grown in complete RPMI-1640 media with 10% (v/v) FBS; THP1 and HEL cultures were supplemented with 1.0 mM sodium pyruvate (Sigma). All cultures contained 1% (v/v) penicillin/streptomycin solution (Invitrogen) and were maintained at 37^{o}C and 5% CO_2. Tissue culture media was obtained from Invitrogen.

Table 3.6 Cell Lines Used in This Study

Cell Line	Leukemia[a]	Reference[b]
HL60	AML (M2)	Dalton et al., 1988.
KG1	AML (M6)	Koeffler and Golde, 1978.
KG1A	AML (M6)	Koeffler et al., 1980.
AML193	AML (M5)	Lange et al., 1987.
THP1	AML (M5)	Tsuchiya et al., 1982.
HEL	AML (M6)	Martin and Papayannopoulou, 1982.
K562	CML (BP)*	Lozzio et al., 1981.

[a] Type of leukemia for each cell line is noted with FAB classification in brackets. The asterisk (*) denotes blast phase (BP), a WHO classification.
[b] Reference for original establishment of cell line or that which is most relevant.

3.5.2. DNA Methylation Inhibitor Treatments

For DNMT inhibitor studies, cells were treated with 4 μM 5-Aza-dC (Sigma) in a time course assay with cell harvesting every 12 hours for four days. For chromatin immunoprecipitation studies, cells were typically treated with 4 μM 5-Aza-dC for 72 hours prior to harvesting as per the chromatin immunoprecipitation protocol. A zebularine (Calbiochem) dose response assay was performed that incorporated 2 to 500 μM of drug treated for 72 hours. Other concentrations of 5-Aza-dC and zebularine treatment are noted in the appropriate

figure(s) and an equivalent volume of 1X PBS was routinely used as a vehicle control.

3.5.3. Histone Deacetylase Inhibitor Treatments

For HDAC inhibitor studies, cells were treated with 1 μM TSA (Sigma) for 12 hours. When TSA was administered in combination with DNMT inhibitors, it was typically treated for the final 12 hours of the 72 hour DNMT inhibition culture period, as noted above. An equivalent volume of ethanol was routinely used as a vehicle control.

3.6. General Molecular Techniques

3.6.1. Agarose Gel Electrophoresis

3.6.1.1. Standard Agarose Gel Electrophoresis

Sample loading buffer consisted of 0.015% (w/v) bromophenol blue, 10% (v/v) glycerol, and 10 mM ethylenediamine tetra-acetic acid (EDTA). Standard DNA fragment gel electrophoresis was typically performed in 2% (w/v) agarose gels in either 1X TAE (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.0) or 1X TBE (90 mM Tris, 90 mM borate, 1 mM EDTA, pH 8.3) buffers containing 0.5 μg/ml ethidium bromide and run between 90 and 110 volts for the appropriate amount of time required for optimal resolution. Gels were visualized under ultraviolet light and either photographed or digitally captured using a gel documentation system (Bio-Rad).

3.6.1.2. Formaldehyde Agarose Gel Electrophoresis

Total RNA samples were routinely checked for ribosomal integrity by formaldehyde agarose gel electrophoresis. Sample loading buffer consisted of 1X MOPS [3-(N-morpholino) propane sulfonic acid], 1.3 mM EDTA, 50% formamide, 18% formaldehyde, 0.015% (w/v) bromophenol blue, 5% (v/v) glycerol, and 0.5 μg/μl ethidium bromide. Samples were prepared for electrophoresis by heating 2 μg of total RNA in two volumes of loading buffer to 70^{o}C for 10 min followed by placement on ice. When cooled, the samples were loaded and run on 1% (w/v) agarose/16% (v/v) formaldehyde gels using 1X MOPS as running buffer at approximately 90 volts for 45 min or until the desired resolution was achieved. Gels were visualized under ultraviolet light and either photographed or digitally captured using a gel documentation system (Bio-Rad).

3.6.2. Generation of 3' End Labeled Oligonucleotide Probes

Terminal deoxynucleotidyl transferase (TdT) is an enzyme that catalyzes the repetitive addition of mononucleotides from deoxynucleotide triphosphates (dNTPs) to the terminal 3´-OH of a DNA initiator, accompanied by the release of inorganic phosphate. End labeled probes were generated by combining 2 pmol of target oligonucleotide (Table 3.4.), 1X TdT buffer [200 mM potassium cacodylate, 25 mM Tris, 0.01% (v/v) Triton X-100, 1 mM $CoCl_2$, pH 7.2], approximately 15 μCi (555 kBq) [α^{32}P]-deoxycytidine-5'-triphosphate (dCTP; Perkin Elmer), and 40 units of rTdT enzyme (Invitrogen), and incubated at 37^{o}C for 30 min. The reaction was stopped by heating at 70^{o}C for 10 min and the radiolabeled probe was added

directly to hybridization solution [10% (w/v) polyethylene glycol-8000, 1% (v/v) sodium dodecyl sulfate (SDS), 5X sodium chloride/sodium citrate (SSC) buffer].

3.6.3. Small Scale Isolation of Plasmid DNA from Bacterial Cells

Isolation and purification of plasmid DNA from bacterial cells for sequencing was prepared using the QIAprep Miniprep kit (Qiagen). The procedure was followed according to the manufacturer's instructions, which typically yielded approximately 5 μg of plasmid DNA per ml of bacterial culture, and samples were stored at -20^{o}C until needed.

3.6.4. Isolation of Total RNA from Eukaryotic Cells

Total RNA was isolated using TRIzol (Invitrogen) which is a modification of the method described by Chomczynski and Sacchi (Chomczynski and Sacchi, 1987). The procedure was followed according to the manufacturer's instructions, which typically yielded approximately 50 μg of total RNA per 10^7 starting cells. RNA pellets were dissolved in nuclease-free water (Ambion) and concentration and purity was determined by standard A_{260}/A_{280} spectrophotometric reading. Samples were stored at -80^{o}C until needed.

3.6.5. Isolation of DNA from Eukaryotic Cells

Genomic DNA was isolated using TRIzol (Invitrogen) and the procedure was followed according to the manufacturer's instructions, which typically yielded 80 - 100 μg of genomic DNA per 10^7 starting cells. DNA pellets were dissolved in

8 mM NaOH and adjusted to approximately pH 8.4 with 0.1 M HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer. Concentration and purity was determined by standard A_{260}/A_{280} spectrophotometric reading and samples were stored at -20^{o}C until needed.

3.6.6. Isolation of Protein Lysates from Eukaryotic Cells

Protein cell lysates were harvested using radioimmunoprecipitation (RIPA) buffer [50 mM Tris-HCl, pH 7.4, 1% (v/v) NP-40, 0.25% (w/v) sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml aprotinin, 1 μg/ml pepstatin A, 1 mM sodium orthovanadate, 1 mM sodium fluoride]. Briefly, cells were washed twice in ice cold 1X PBS and cell pellets were resuspended in ice cold RIPA buffer (0.5 ml per 10^7 cells) and incubated on ice for 15 min. Samples were centrifuged at 4^{o}C for 15 min at 12000 x *g*, supernatants transferred to new tubes, and protein concentration was determined by the Bradford assay as per the manufacturer's instructions (Bio-Rad). The procedure typically yielded approximately 200 μg of protein per 10^7 starting cells and samples were stored at -80^{o}C until needed.

3.6.7. Isolation of Histone Proteins from Cultured Eukaryotic Cells

Protein cell lysates enriched for nuclear histones were harvested essentially as described elsewhere (Nguyen et al., 2002). Briefly, cell pellets were lysed in an isolation buffer consisting of 10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol (DTT), 1.5 mM PMSF, and 0.2 M HCl (added immediately

prior to incubation). Following incubation on ice for 30 min and centrifugation at 4°C for 15 min at 12000 x *g*, the supernatant was buffer exchanged with twenty volumes of the above isolation buffer (without HCl) and concentrated using Amicon centrifugal filtration units with a nominal molecular weight limit of 10 KDa (Millipore). Protein concentration was determined by the Bradford assay as per the manufacturer's instructions (Bio-Rad) and samples were stored at -80°C until needed.

3.7. Reverse Transcriptase PCR (RTPCR)

Total RNA was used as a template for the synthesis of cDNA using the Moloney murine leukemia virus reverse transcriptase (M-MLV-RT) system according to the manufacturer's instructions (Promega). Briefly, 2 µg of total RNA was added to a mix containing 1X M-MLV-RT buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$), 10 mM DTT, 1.25 mM of each dNTP, 500 ng random hexamers (Fermentas), 20 units of RNasin (Promega), and 200 units of M-MLV-RT in a final volume of 20 µl. Synthesis of cDNA was completed by incubation at 37°C for one hour and samples used immediately or stored at -20 °C.

PCR was performed in a final volume of 30 µl containing 1.0 µl of cDNA template, 1x REDTaq PCR Reaction Buffer (Sigma), 2.5 mM $MgCl_2$, 0.4 mM of each dNTP, 0.2 µM of each primer, and 1.5 units of REDTaq DNA Polymerase (Sigma). The amplification consisted of an initial denaturation step at 95°C for 3 min followed by 30 amplification cycles (94°C for 45 sec, annealing temperature for 45 sec, and 72°C for 1 min) and a final incubation at 72°C for 10 min. Primer

sequences and annealing temperatures are indicated in Table 3.4. Following amplification, 10 μl of individual PCR products were visualized by standard agarose gel electrophoresis without the addition of bromophenol blue loading buffer. Semiquantitation of target gene mRNA was analyzed by densitometry using Quantity One imaging software (Bio-Rad) of the appropriately sized RTPCR product normalized to that of the corresponding samples β-Actin housekeeping gene RTPCR product. Differences in target gene mRNA following drug treatment were calculated by dividing the target genes' drug treated normalized value by the untreated control normalized value and articulated numerically as 'fold change'.

3.8. RNase Protection Assay (RPA)

RPA was carried out with the BD RiboQuant RPA system (BD Biosciences) according to the manufacturer's instructions using a probe set with templates of distinct length for the CDKIs p21 and p27, and for the ubiquitously expressed housekeeping genes L32 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Briefly, synthesis of high-specific activity [α^{32}P]-uridine triphosphate (UTP; Amersham) labeled antisense RNA probes was accomplished using T7 polymerase and the probes were hybridized in excess to 10 μg of total RNA isolated from patient samples and cell lines. Following this, free probe and other single-stranded RNA species were digested with RNase and the remaining RNase-protected probes were purified, resolved by denaturing polyacrylamide gel electrophoresis [4.75% (v/v) acrylamide:N,N'-methylene-bis-acrylamide (19:1), 8 M urea] in 0.5X TBE running buffer, and visualized by autoradiography using

Hyperfilm MP (Amersham). The intensity of appropriately-sized, protected probe fragments corresponding to L32 and GAPDH gene transcripts was used to ensure equivalent RNA loading among the different samples.

3.9. DNA Methylation Analysis

3.9.1. Sodium Bisulfite Treatment of Genomic DNA

Genomic DNA was treated with sodium bisulfite reagent which converts unmethylated cytosine residues to uracil, and eventually thymidine following PCR, for downstream methylation analysis essentially as described elsewhere (Herman et al., 1996a; Tao et al., 2002). Briefly, 10 μg of DNA was incubated at 37°C for 15 min in 0.3 N NaOH. Samples were then mixed with 2.2 M sodium bisulfite/0.5 mM hydroquinone reagent (pH 5.0) and incubated at 55°C in the dark for 6 - 8 hours. Following incubation, modified DNA was purified using a Wizard DNA Clean-up column (Promega) as per the manufacturer's instructions and eluted in 45 μl of pre-warmed nuclease-free water (Ambion). Modification was completed by desulfonation with 0.3 N NaOH at 37°C for 15 min and precipitation with 3 M ammonium acetate and overnight ethanol incubation at -20°C. Modified DNA pellets were washed with 75% (v/v) ethanol, resuspended in 50 μl of water, and used immediately or stored at -20°C until needed.

3.9.2. Methylation Specific PCR (MSP)

DNA methylation within promoter associated CpG islands was determined by MSP following sodium bisulfite treatment of genomic DNA as above. PCR was

performed in a final volume of 40 µl containing 100 - 200 ng of bisulfite-treated DNA, 1x Qiagen PCR Buffer, 1.5 - 3.5 mM $MgCl_2$, 0.4 mM of each dNTP, 0.2 µM of each primer set, and 0.2 units of HotStar *Taq* (Qiagen). The amplification consisted of a *Taq* activation step at 95°C for 15 min followed by 35 amplification cycles (94°C for 1 min, annealing temperature for 1 min, and 72°C for 1.5 min) and a final incubation at 72°C for 10 min. The 5' CpG islands were identified using the online 'CpG Island Searcher' tool (Takai and Jones, 2003) and appropriate primers were designed near the target genes major transcriptional start site using Vector NTI software (Invitrogen) or the online 'MethPrimer' tool (Li and Dahiya, 2002). The primer sequences and optimal annealing temperatures are listed in Table 3.4. CpGenome universally methylated DNA (Chemicon), following bisulfite modification, served as the methylated MSP positive control.

3.9.3. Temporal Temperature Gradient Gel Electrophoresis (TTGE)

CpG island hypermethylation within target gene promoters was also determined by TTGE. In this analysis, bisulfite-treated DNA is amplified with gene specific primers that amplify both unmethylated and methylated promoter sequences concurrently. Differentially methylated sequences within the PCR product are then resolved according to differences in melting temperatures (Guldberg et al., 2002). WinMelt software (Bio-Rad) was used to generate the melting profiles of unmethylated and methylated CpG promoter sequences. Vector NTI software (Invitrogen) was used to select primers specific for bisulfite-treated DNA to amplify regions within the CpG islands of target genes near transcriptional

starting sites. In order to minimize amplification of genomic sequences not converted by bisulfite treatment and to avoid biased amplification of methylated alleles, primers were designed to contain the maximum number of uracils converted from cytosines and to lack CpG dinucleotides. 5'-GC-clamps were attached to all reverse primers to enhance separation of unmethylated and methylated alleles by TTGE and primer sequences and optimal annealing temperatures are listed in Table 3.4. PCR was performed in a final volume of 40 µl containing 100 - 200 ng of bisulfite-treated DNA, 1x Qiagen PCR Buffer, 3.5 mM $MgCl_2$, 0.4 mM of each dNTP, 0.2 µM of each primer set, and 0.2 units of HotStar *Taq* (Qiagen). The PCR reactions consisted of an initial *Taq* activation step of 15 min at 95°C, followed by 35 amplification cycles (94°C for 1 min, annealing temperature for 1 min, and 72°C for 1.5 min) and a final incubation at 72°C for 10 min.

PCR products were electrophoresed in polyacrylamide gels [p15: 8%; p21: 6%; p27: 6% (v/v) acrylamide:N,N'-methylene-bis-acrylamide (37.5:1)] containing urea (p15: 6 M; p21: 7 M; p27: 6 M), 1.25X TAE, and 0.1% (v/v) ammonium persulfate (APS). Gels were polymerized by the addition of 0.04% (v/v) N,N,N,N-tetramethyl-ethylenediamine (TEMED) and run at 130 volts in 1.25X TAE buffer over a specific temperature range (p15: 53 - 63°C; p21: 52 - 64°C; p27: 56 - 66°C) and at a specific temperature ramp rate (p15: 2.0°C/hour; p21: 1.5°C/hour; p27: 1.7°C/hour) using a DCODE temperature controlling vertical gel apparatus (Bio-Rad). Gels were then transferred and fixed to nylon membranes (Amersham), hybridized overnight to [α^{32}P]-dCTP labeled oligonucleotide probes (see Section

3.5.2 and Table 3.4) specific for the target gene and the resulting differentially methylated PCR products visualized by autoradiography using Hyperfilm MP (Amersham). Plasmids containing representative unmethylated or methylated sequences were constructed from previously characterized hematological malignancy samples and their differentially methylated sequences confirmed by cloning and sequencing (see Section 3.9.2). These plasmids were used as control PCR templates to assess TTGE migration within the unknown AML samples.

3.10. DNA Sequencing

3.10.1. Direct PCR Product Sequencing

PCR products were purified using a QIAquick PCR Purification kit (Qiagen) as per the manufacturer's instructions. Pure samples were quantitated by comparison to DNA markers (Fermentas) following standard agarose gel electrophoresis and automated DNA sequencing was performed with the forward PCR primer by Annette Kerviche at the Saskatchewan Cancer Agency DNA Sequencing Facility using an ABI Prism 310 Genetic Analyzer.

3.10.2. TTGE Band Recovery and Sequencing

Cloning and sequencing of individual bands from TTGE polyacrylamide gels was performed by first purifying DNA fragments using a procedure adapted from the QIAquick Gel Extraction Protocol (Qiagen) and from Sambrook et al. (Sambrook et al., 1989). Briefly, individual bands from TTGE gels were scalpel excised and slices were added to two volumes of diffusion buffer [0.5 M

ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, pH 8.0, 0.1% (v/v) SDS] and incubated at 50°C for 30 min. Following 10000 x *g* centrifugation for 2 min, the supernatant was removed and processed with a QIAquick Gel Extraction column (Qiagen) as per the manufacturer's instructions. DNA was eluted in 30 µl of elution buffer and 2 µl was inserted into a pCR2.1-TOPO vector using a TOPO TA cloning kit (Invitrogen) and transformed into competent *Escherichia coli* as per the manufacturer's instructions. Selective Luria-Bertani (LB)-agar plates for isolation of transformed colonies consisted of LB broth [1.0% (w/v) tryptone, 0.5% (w/v) yeast extract, 1.0% (w/v) NaCl, pH 7.0] with 1.5% (w/v) agar and 50 µg/ml ampicillin. Plates were incubated overnight upside-down at 37°C. Isolated, transformed, bacterial colonies were picked from LB-ampicillin plates and transferred to LB-ampicillin liquid culture media and grown overnight. Bacteria were then pelleted and plasmid DNA isolated as described above (Section 3.5.3.). Purified samples were quantitated by standard A_{260}/A_{280} spectrophotometric reading and automated DNA sequencing was performed with the M13 Forward (-20) primer (Invitrogen) by Annette Kerviche at the Saskatchewan Cancer Agency DNA Sequencing Facility using an ABI Prism 310 Genetic Analyzer.

3.11. Chromatin Immunoprecipitation (ChIP)

Following culture, formaldehyde was added directly to the cell media to a final concentration of 1%, and cells were incubated at room temperature for 10 min with agitation. The cross-linking reaction was quenched by the addition of 125 mM glycine and agitated for another 5 min at room temperature. Cells were

washed twice in ice cold 1X PBS and lysed in 350 μL of Upstate lysis buffer supplemented with freshly added protease inhibitors (1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml pepstatin A). Lysate genomic DNA was sheared to lengths between 200 and 800 bp by sonication (Branson Sonifier 450, 1.5 minutes, 1.5 output control, 60% duty cycle). Sheared lysates were cleared by centrifugation, and 50 μL was set aside as an 'input' PCR positive control fraction. The remaining lysate was split: one half [Ab (+)] was immunoprecipitated with a specific antibody (anti-MeCP2: 8.0 μg; anti-SUV39h1: 10 μg; anti-HDAC1: 4.0 μg; anti-H3-acetyl-K9/14: 4.0 μg; anti-H3-acetyl-K18: 8.0 μg; anti-H3-acetyl-K23: 8.0 μg; anti-H3-dimethyl-K4: 4.0 μg; anti-H3-dimethyl-K9: 8.0 μg; anti-H3-dimethyl-R17: 8.0 μg; anti-H4-acetyl-K5/8/12/16: 4.0 μg; all from Upstate Biotechnology), and the other half [Ab (-)] was immunoprecipitated with pre-immune rabbit IgG. Both fractions were diluted and processed with buffers and reagents supplied with the ChIP Assay kit according to the manufacturer's instructions (Upstate Biotechnology).

Following the ChIP protocol, cross-links from all samples (including the input fraction) were reversed by heating and genomic DNA was isolated by proteinase K digestion, phenol extraction, and ethanol precipitation with the assistance of 15 μg of GlycoBlue coprecipitant (Ambion). DNA pellets from all samples were dissolved in 40 μl of water and used immediately or stored at -20°C until needed. PCR amplification was performed on diluted input, Ab (-), and Ab (+) DNA samples in 1x Qiagen PCR Buffer, 1.5 - 2.8 mM $MgCl_2$, 0.4 mM of each dNTP, 0.2 μM of each primer set, and 0.2 units of HotStar *Taq* (Qiagen). The amplification consisted of an initial *Taq* activation step at 95°C for 15 min followed

by 35 amplification cycles (94°C for 1 min, annealing temperature for 1 min, and 72°C for 1.5 min) and a final incubation at 72°C for 10 min. The primer sequences and optimal annealing temperatures are listed in Table 3.4. Following PCR, all amplification products were visualized by standard agarose gel electrophoresis and analyzed by semi-quantitative densitometry using Quantity One imaging software (Bio-Rad).

3.12. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Protein lysates, histone enriched lysates, and prestained protein molecular weight markers (Fermentas) were resolved on SDS-PAGE gels. Samples were heated at 95°C for 5 min in equal volumes of 2X sample buffer [150 mM Tris-HCl, pH 6.8, 1.2% (v/v) SDS, 30% (v/v) glycerol, 15% (v/v) β-mercaptoethanol, 1.8% (w/v) bromophenol blue] and immediately placed on ice. Following a brief cooling period, samples were loaded onto gels typically containing 15% (v/v) acrylamide:N,N'-methylene-bis-acrylamide (37.5:1), 380 mM Tris-HCl, pH 8.8, 0.1% (v/v) SDS and 0.1% (v/v) APS in the resolving gel, and 3% (v/v) acrylamide:N,N'-methylene-bis-acrylamide (37.5:1), 125 mM Tris-HCl, pH 6.8, 0.1% (v/v) SDS and 0.1% (v/v) APS in the stacking gel. The percentage of acrylamide in the resolving gels was adjusted based on the molecular weight of the target protein to be resolved. Gels were polymerized by the addition of 0.04% TEMED and run in buffer containing 25 mM Tris-HCl, pH 8.3, 190 mM glycine, and 0.1% (v/v) SDS at 175 volts at room temperature until the loading dye reached the end of the resolving gel. Parallel gels were routinely stained with coomassie

blue staining solution [0.25% (w/v) Coomassie Brilliant Blue R-250 (Bio-Rad), 45% (v/v) methanol, 10% (v/v) acetic acid] and destained [45% (v/v) methanol, 10% (v/v) acetic acid] to ensure equivalent sample loading and protein resolution.

3.13. Western Blotting

Resolved proteins in SDS-PAGE gels were transferred to nitrocellulose membranes (Bio-Rad) using a Bio-Rad Trans-Blot SD Semi-Dry Transfer Cell run at 15 volts for 20 min at room temperature in transfer buffer containing 25 mM Tris-HCl, pH 8.3, 192 mM glycine, 20% (v/v) methanol, and 0.1% (v/v) SDS. Following transfer, membranes were washed in water and blocked for 20 - 60 min at room temperature with constant agitation in either PBS or TBS based blocking buffer containing non-fat dry milk (Carnation) according to the primary antibody manufacturer's instructions. After blocking, membranes were incubated with the primary antibody diluted appropriately in the requisite blocking buffer. Typically, membranes were incubated with the primary antibody overnight at $4^{o}C$ with constant agitation. Membranes were then washed and incubated with the appropriate dilution of secondary antibody in blocking buffer. The primary and secondary antibodies used in the study are listed in Table 3.5. After incubation with the secondary antibody solution, membranes were washed and antibody-protein complexes visualized using the Immun-Star HRP Chemiluminescent kit (Bio-Rad) and Hyperfilm MP (Amersham).

3.14. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] Assay

Cell survival/cytotoxicity was measured by the MTT assay. Cells were plated in triplicate at 2 x 10^4 cells per well in a 96 well plate, cultured as above, and treated with 1 or 4 μM 5-Aza-dC, 250 or 500 μM zebularine, or a vehicle control. Following the 72 hour treatment period, 1/10 culture volume of 5 mg/ml MTT labeling reagent (Sigma) was added to each well and cultured for an additional 4 hours. The resulting formazan crystals were solubilized by the addition of 100 μl of solubilization solution [10% (v/v) SDS, 0.01 M HCl] to each well and cultured overnight prior to spectrophotometric absorbance reading (570nm with 660nm background subtraction) using a SpectraMax microplate reader (Molecular Devices).

3.15. Tritiated Thymidine Incorporation Assay

Cell proliferation was measured by the tritiated [^{3}H]-thymidine incorporation assay. Cells were plated in quadruplicate at 5 x 10^4 cells per well in a 96 well plate, cultured as above, and treated with 1 or 4 μM 5-Aza-dC, 250 or 500 μM zebularine, or a vehicle control. For the final 6 hours of the 72 hour treatment period 0.5 μCi (18.5 kBq) of [^{3}H]-thymidine (Amersham) was added to each well of the experiment. Cells were harvested and liquid scintillation counting was used to measure the [^{3}H]-thymidine that was incorporated into newly synthesized DNA, directly reflecting the degree of cell proliferation.

3.16. Propidium Iodide (PI) Staining

Cell cycle analysis was performed by PI staining of DNA content. Cells were cultured as above, and treated with 1 or 4 μM 5-Aza-dC, 250 or 500 μM zebularine, or a vehicle control. Briefly, 2 x 10^6 cells (in triplicate) were washed in ice cold 1X PBA [1X PBS, 0.1% (w/v) bovine serum albumin, 0.02% (w/v) sodium azide] and fixed in ice cold ethanol. Cell pellets were reconstituted in Triton-PBA [0.1% (v/v) Triton X-100, 1X PBA] for 3 min, pelleted again by centrifugation, and incubated in 500 units/ml RNase working solution (Worthington) at 37°C for 20 min. Samples were stained overnight in 2 ml PI working solution (0.05 mg/ml in PBA; Sigma) at 4°C and filtered through 35 μM nylon mesh into glass tubes for flow cytometry analysis.

3.17. cDNA Microarray Analysis

This experiment was performed in collaboration with Dr. Derek S. Pearson and Matthew N. Bainbridge at the University of Saskatchewan. Dr. Pearson participated in the experiment by repeating the human 1.7K arrays using previously harvested RNA samples as described above, and an indirect labeling reverse transcriptase methodology essentially as described by the University Health Network Microarray Centre (http://www.microarray.ca/). Mr. Bainbridge assisted in the experiment by combining the data sets from each array labeling methodology (direct and indirect) and analyzing the data using Acuity 3.1 bioinformatics software (Molecular Devices).

3.17.1. Sample Preparation

Total RNA from each culture condition was isolated as described above and 20 - 30 μg was purified using the RNase-Free DNase and RNeasy Mini kits as per the manufacturer's instructions (Qiagen). Samples were eluted with the supplied RNase-free water and concentration and purity was determined by standard A_{260}/A_{280} spectrophotometric reading. Samples were used immediately or stored at -80°C until needed.

3.17.2. Direct Labeling with Cyanine 3- and Cyanine 5-dCTP

Purified RNA samples from each culture condition were reverse transcribed using fluorescent Cyanine-labeled dCTP as follows. Briefly, 15 μg of total RNA was combined with 1X Superscript First Strand reaction buffer (Invitrogen), 10 mM DTT, 0.5 mM each of dATP, dGTP and dTTP, 0.05 mM dCTP, 150 pmol Oligo-dT_{12-18} primer (Invitrogen), 5.0 ng control *Arabidopsis thaliana* RNA, and 25 μM of either Cyanine 3- or Cyanine 5-dCTP (NEN Life Sciences) in a final volume of 40 μl. The control and experimental labeling reactions were incubated at 65°C for 5 min and 42°C for 5 min in the dark prior to the direct addition of SuperScript II reverse transcriptase (400 units; Invitrogen) and RNasin (40 units; Promega). Labeling proceeded at 42°C for 3 hours in the dark and was stopped by placement on ice, the addition of 4 μl of 50 mM EDTA (pH 8.0) and 2 μl of 10 N NaOH, and incubation at 65°C for 20 min. Following an addition of 4 μl of 5 M acetic acid, the control and experimental samples were combined and precipitated at -20°C for 30 min with 100 μl isopropanol. Samples were centrifuged for 10 min at 14000

rpm at 4°C and pellets were washed in 70% ethanol. Pellets containing both control and experimental cDNA were briefly dried at room temperature and resuspended in 10 µl 1X TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Samples were then mixed with 40 µl of hybridization solution [1X DIG Easy Hyb solution (Roche), 50% (w/v) yeast tRNA (Invitrogen), 50% (w/v) calf thymus DNA (Sigma), heated at 65°C for 2 min and cooled to room temperature] and incubated at 65°C for 2 min and cooled to room temperature.

3.17.3. Probe Hybridization

Hybridization mixtures (50 µl) were directly pipetted onto 22 x 40 mm Hybri-slips (Sigma) followed by careful placement, array side down, of human 1.7K arrays (Microarray Centre) containing 1718 known ESTs. Slides were carefully enclosed in a hybridization chamber (Genetix) containing a small volume (approximately 2 ml) of DIG Easy hybridization solution and incubated on a level surface at 37°C for approximately 18 hours.

3.17.4. Microarray Washing

Following hybridization, Hybri-slips were removed by slide immersion in 1X SSC and washed for three sets of 15 min each in 50°C pre-warmed 1X SSC/0.1% (v/v) SDS with occasional gentle agitation. Slides were then rinsed briefly in 1X SSC and 0.1X SSC and carefully spun dry at 600 rpm for 5 min. Arrays were scanned immediately or stored at room temperature in the dark until needed.

3.17.5. Microarray Scanning

Microarray slides were scanned using GenePix Pro 4.1 software and an Axon 4000B scanner (Molecular Devices). Individual 16-bit TIFF images were obtained by scanning each of the two fluors and an overlay image of the two images was created and quantified using GenePix Pro 4.1 software.

3.17.6. Data Analysis

Scanned images and the associated quantification data files were entered into a GeneTraffic Microarray Database and Analysis System (Iobion Informatics) as well as Acuity 3.1 bioinformatics software (Molecular Devices). Individual array spots from each of the three hybridizations had to pass a number of quality criteria to be included in the data analysis and each array dataset was normalized using LOWESS (locally weighted regression scatter plot smoothing) subarray normalization (Quackenbush, 2002). Resultant normalized $\log_2$ ratios were used for statistical analysis.

4. RESULTS AND DISCUSSION

4.1. METHYLATION STATUS OF CDKI GENES IN HUMAN AML

4.1.1. CDKI Expression in Human AML Cell Lines and Patient Samples

Cyclins, CDKs, and CDKIs are frequently deregulated in cancer. As such, absent or reduced CDKI expression is a characteristic of many different tumors including some human leukemias and lymphomas (Drexler, 1998; Tsihlias et al., 1999). Moreover, disruption of the TGF-β1 pathway has been implicated in the pathogenesis of hematological malignancies (DeCoteau et al., 1997; Lowsky et al., 2000) and the CDKIs p15, p21 and p27 are downstream effectors of TGF-β1 signaling (Robson et al., 1999). In order to evaluate CDKI expression in human AML, RTPCR and RPA assays were employed to measure p15, p21, and p27 mRNA in AML cell lines and patient samples.

The p15 gene was evaluated by RTPCR using primers specific for the gene's coding sequence. Interestingly, the assay failed to detect p15 mRNA in 6/7 (86%) AML cell lines (Fig. 4.1A), only being detected in the cell line HL60. This data is consistent with other studies that have analyzed HL60 p15 mRNA by RTPCR (Cameron et al., 1999b; Herman et al., 1996b). Extending this analysis to AML patient material, p15 mRNA was absent in 25/32 (78%) cases (Fig. 4.1B),

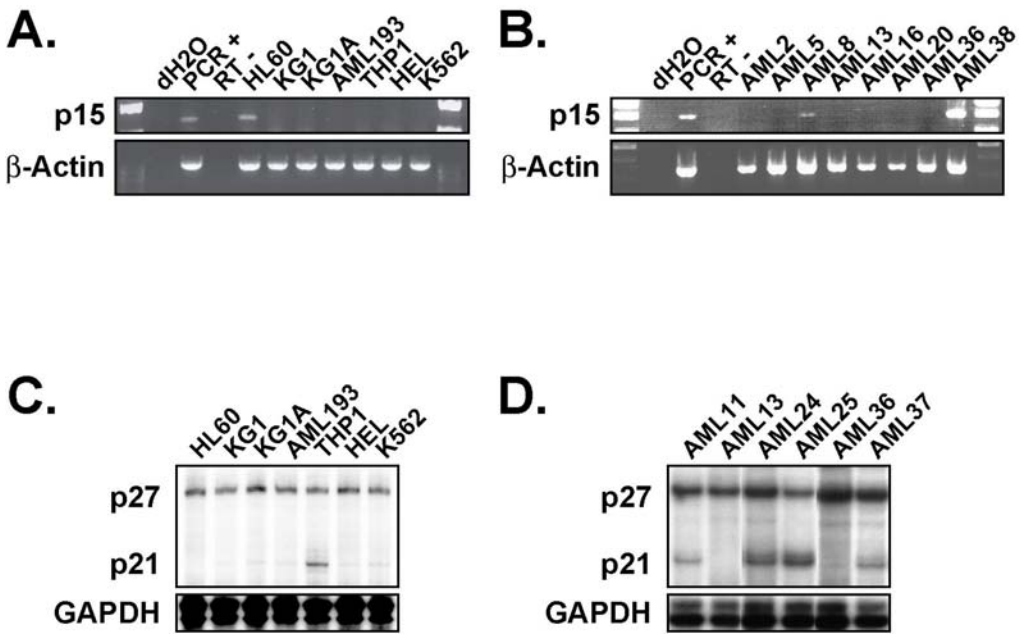

Figure 4.1 CDKI Expression in Human AML. A) and **B)** Representative p15 RTPCR data from AML cell lines **(A)** and patient samples **(B)**. Included in each amplification is a PCR positive control (PCR +), and water and reverse transcriptase negative controls (dH2O and RT -). The housekeeping gene β-Actin was amplified in all cell lines and patient samples to ensure RNA integrity and equivalent cDNA template quantity between samples. Note the presence of detectable p15 mRNA in the cell line HL60 and patients AML8 and AML38. **C)** and **D)** Representative p21 and p27 RPA data from AML cell lines **(C)** and patient samples **(D)**. The RPA template probe set used in this study allowed the simultaneous analysis of p21 and p27, and the housekeeping gene GAPDH within the same reaction ensuring RNA integrity and equivalent RNA loading between samples. Note the presence of detectable p21 mRNA in the cell line THP1 and patients AML11, AML24, AML25 and AML37, and detectable p27 in all cell lines and patients tested.

suggesting the possibility of p15 transcriptional silencing in the molecular pathogenesis of the disease. As a control for cDNA integrity and equivalent RTPCR template quantity, the housekeeping gene β-Actin was amplified in all cell lines and patients analyzed.

The CDKIs p21 and p27 were evaluated by RPA, a sensitive detection method that unlike standard RTPCR methods does not employ an amplification step and therefore provides linear mRNA signals over a broad input range. Another advantage of RPA is its capacity to simultaneously quantify several mRNA species in a single sample of total RNA, which permitted the comparison of p21 with the expression of its CIP/KIP family member p27. Furthermore, incorporating a probe for the housekeeping gene GAPDH allowed comparison of individual CDKI mRNAs between samples. By RPA, p21 and p27 mRNA was absent in 6/7 (86%; only detectable in THP1) and 0/7 (0%) AML cell lines, respectively (Fig. 4.1C). Due to limited RNA availability, RPA analysis was performed on 24 AML patients and p21 and p27 mRNA were absent in 6/24 (25%) and 0/24 (0%) cases, respectively (Fig. 4.1D), suggesting the possibility of p21 transcriptional silencing, and not that of p27, in the molecular pathogenesis of the disease. Combining the AML cell line and patient data, p15, p21, and p27 mRNA were absent in 31/39 (79%), 12/31 (39%), and 0/31 (0%) of cases, respectively.

4.1.2. p15 Promoter Hypermethylation in Human AML

Previous work in our lab has established a correlation between p15 promoter hypermethylation and a lack of detectable p15 mRNA in T-cell

lymphoblastic lymphoma/leukemia (Scott et al., 2004) and others have reported similar findings in a variety of hematological malignancies, including myeloid leukemias (Cameron et al., 1999b; Herman et al., 1997; Toyota et al., 2001). As p15 mRNA was absent in 79% of the AML samples analyzed in this study, the possibility of promoter hypermethylation induced p15 transcriptional silencing was therefore investigated. Two techniques were employed to analyze the degree of p15 promoter methylation: standard MSP and a novel method that resolves differentially methylated promoter alleles on denaturing polyacrylamide gels known as TTGE. Primers and probes for these techniques were designed to be located within the p15 promoter associated CpG island and near the major transcriptional start site (GenBank Accession Number S75756) as illustrated in Figure 4.2A.

MSP analysis of the p15 promoter region in the AML cell lines using previously defined unmethylated and methylated specific primers (Herman et al., 1996a) detected the presence of methylated alleles in KG1, KG1A, and AML193 cells when compared to the methylated positive control (Fig. 4.3A). Unmethylated alleles were detected in HL60 and KG1 cells and neither the unmethylated nor the methylated MSP reactions successfully amplified in THP1, HEL, and K562 cells. The lack of p15 promoter region MSP amplification in THP1, HEL, and K562 cells, although not conclusive, is indicative of gene deletion at this locus, a genetic abnormality known to occur in a small percentage of hematological cancers (Drexler, 1998). The identification of p15 promoter hypermethylation by MSP in KG1 and KG1A cells has been reported previously (Dodge et al., 2001; Herman

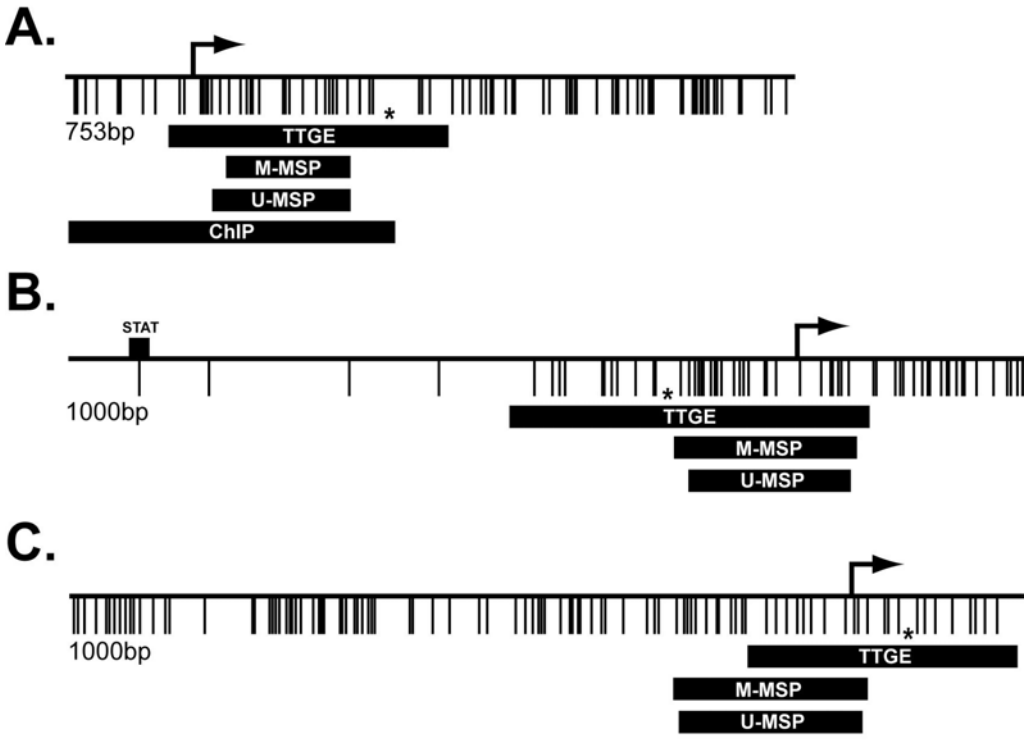

Figure 4.2 p15, p21, and p27 MSP, TTGE, and ChIP PCR Product Locations. Illustrated are the CpG island associated promoter regions of the p15 **(A)**, p21 **(B)**, and p27 **(C)** genes and the locations of amplification products (labeled horizontal black bars) used in this study. Arrow heads represent the location of the major transcriptional start sites, vertical black lines represent CpG dinucleotides, and asterisks (*) denote the location of TTGE methylation-independent probe oligonucleotide binding sites. STAT: Signal Transducer and Activator of Transcription.

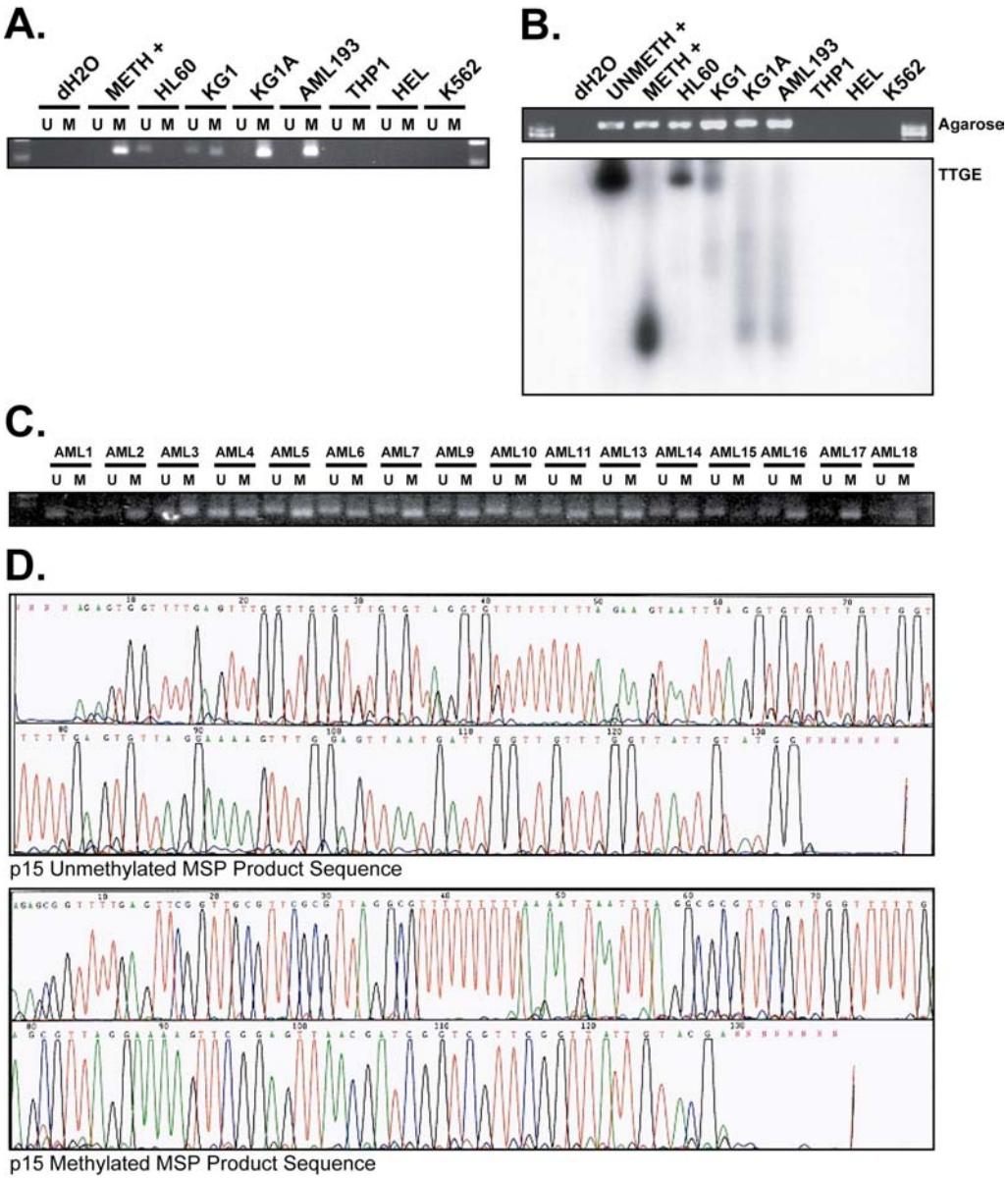

Figure 4.3 p15 Methylation in Human AML. **A)** MSP analysis of bisulfite modified AML cell line DNA. Commercially available universally methylated bisulfite modified DNA was used as a methylated positive control (METH +) and water as a negative reagent control (dH2O). **B)** TTGE analysis of 30 CpG sites within the p15 promoter in AML cell lines. Differentially methylated sequences within the p15 amplicons were resolved according to differences in melting temperature and compared to the known controls (UNMETH + and METH +). **C)** MSP analysis of bisulfite modified AML patient sample DNA. **D)** Representative MSP product sequences from both the unmethylated and methylated PCR reactions. U: unmethylated MSP reaction; M: methylated MSP reaction.

et al., 1996b), yet this is the first study to our knowledge to report p15 promoter hypermethylation in AML193 cells.

To confirm the p15 MSP cell line data, TTGE analysis was performed incorporating known p15 unmethylated and methylated positive controls. As illustrated in Figure 4.3B, TTGE analysis corroborated the MSP data whereby HL60 cells amplified only unmethylated p15 alleles, KG1 cells amplified both unmethylated and methylated alleles, and KG1A and AML193 cells amplified strictly methylated p15 alleles. Of note, all successful TTGE PCR reactions amplify products of equal size, regardless of methylation status, as visualized by standard agarose gel electrophoresis, and only following TTGE do the differentially methylated products become detectable. Again, similar to the MSP data, this locus was unable to amplify with TTGE PCR primers in THP1, HEL, and K562 cells.

Bisulfite treated DNA from the 32 AML patients that were analyzed for p15 mRNA expression and that from another 8 AML samples was available for MSP analysis and altogether 36/40 (90%) cases had detectable p15 promoter hypermethylation (Fig. 4.3C). Of note, no methylation of the p15 gene was detected in the DNA of 13 non-leukemic controls (data not shown). To ensure that the amplification of bisulfite treated DNA was specific for each MSP primer set, representative samples from both the unmethylated and methylated reactions were directly sequenced using the appropriate forward primer (Fig. 4.3D). Importantly, the sequences confirm that no methylation is present within the amplification product of the unmethylated specific MSP primers and that CpG dinucleotides, representing 5-methyl-CpG in the native DNA, are present within the amplification

product of the methylated specific MSP primers. Taken together, p15 promoter hypermethylation was identified in 39/47 (83%) AML cell lines and patient samples.

4.1.3. p21 Promoter Hypermethylation in Human AML

Mutations and deletions of p21 occur only rarely in human cancer but the gene is frequently repressed by epigenetic mechanisms (Ying et al., 2004). However, investigation of hematological malignancies and solid tumors for p21 promoter hypermethylation, an important mechanism of epigenetic silencing, have shown variable, and in some cases, discrepant results (Roman-Gomez et al., 2002; Shen et al., 2002; Ying et al., 2004). As p21 mRNA was absent in 39% of the AML samples analyzed in this study, the possibility of promoter hypermethylation induced p21 transcriptional silencing was therefore investigated. Two techniques were employed to analyze the degree of p21 promoter methylation: standard MSP and TTGE. Primers and probes for these techniques were designed to be located within the p21 promoter associated CpG island and near the major transcriptional start site (GenBank Accession Number U24170) as illustrated in Figure 4.2B.

MSP analysis of the p21 promoter region did not detect methylated alleles in any of the AML cell lines when compared to the methylated positive control (Fig. 4.4A). In contrast, unmethylated alleles were detected in all of the cell lines tested despite the fact that only the cell line THP1 had detectable mRNA by RPA. These results were confirmed by TTGE, as shown in Figure 4.4B, whereby all AML cell line TTGE PCR products migrate parallel to the unmethylated control.

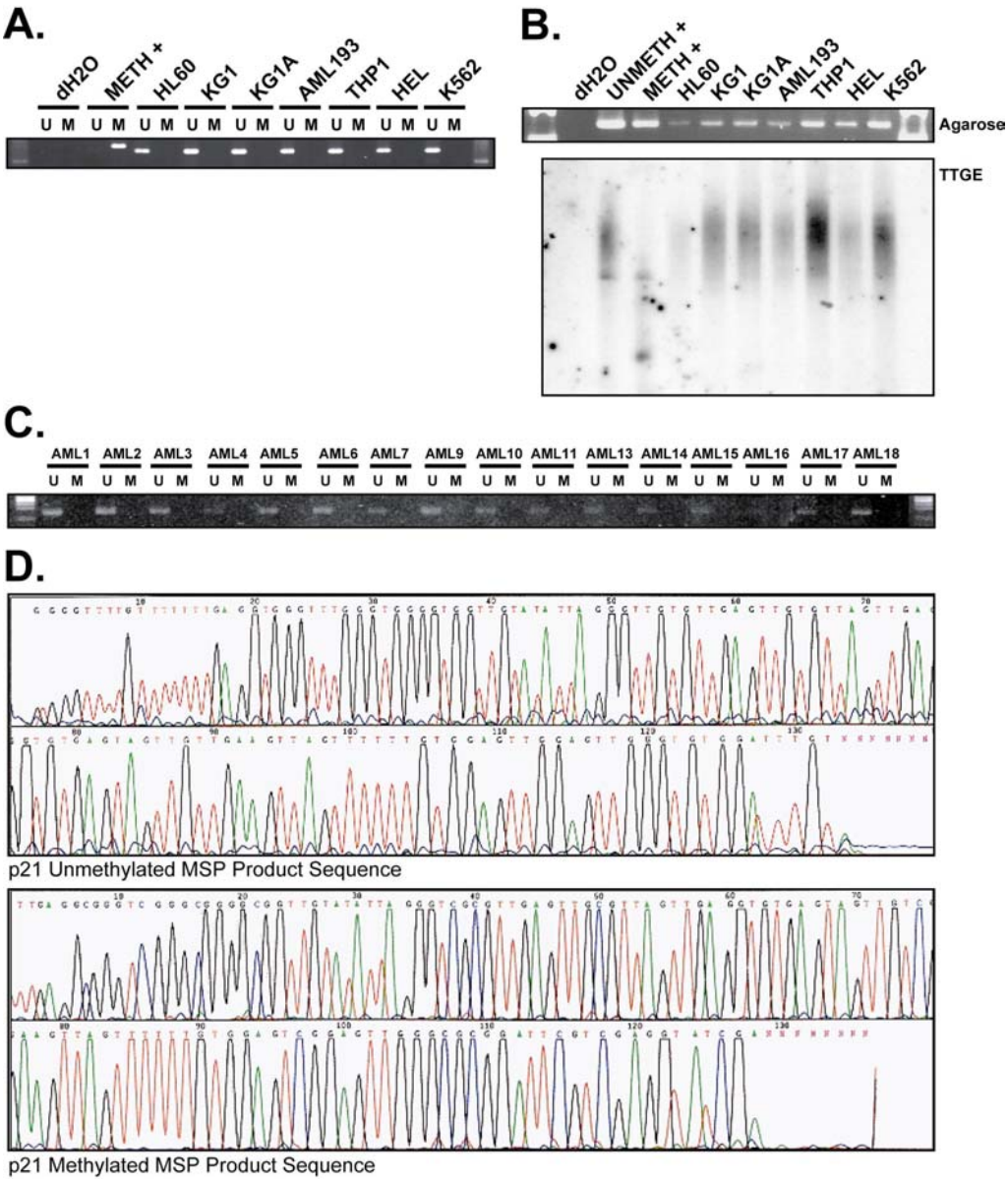

Figure 4.4 p21 Methylation in Human AML. **A)** MSP analysis of bisulfite modified AML cell line DNA. Commercially available universally methylated bisulfite modified DNA was used as a methylated positive control (METH +) and water as a negative reagent control (dH2O). **B)** TTGE analysis of 35 CpG sites within the p21 promoter in AML cell lines. Differentially methylated sequences within the p21 amplicons are resolved according to differences in melting temperature and compared to the known controls (UNMETH + and METH +). **C)** MSP analysis of bisulfite modified AML patient sample DNA. **D)** Representative MSP product sequences from both the unmethylated and methylated PCR reactions. U: unmethylated MSP reaction; M: methylated MSP reaction.

Bisulfite treated DNA from the 24 AML patients that were analyzed for p21 mRNA expression and that from another 16 AML samples was available for MSP analysis and altogether 0/40 (0%) cases had detectable p21 promoter hypermethylation (Fig. 4.4C). Moreover, no methylation of the p21 gene was detected in the DNA of 13 non-leukemic controls (data not shown). To ensure that the amplification of bisulfite treated DNA was specific for each MSP primer set, representative samples from both the unmethylated and methylated reactions were directly sequenced using the appropriate forward primer (Fig. 4.4D). Importantly, the sequences confirm that no methylation is present within the amplification product of the unmethylated specific MSP primers and that CpG dinucleotides, representing 5-methyl-CpG in the native DNA, are present within the amplification product of the methylated specific MSP primers. Taken together, p21 promoter hypermethylation was identified in 0/47 (0%) AML cell lines and patient samples.

4.1.4. p27 Promoter Hypermethylation in Human AML

p27 promoter hypermethylation has been reported in some subtypes of hematological malignancies, with lymphoma cases reported to be positive for p27 methylation in association with absent p27 protein (Go, 2003; Kibel et al., 2001; Nakatsuka et al., 2003). Although all samples analyzed in this study had detectable p27 mRNA by RPA, the presence of p27 promoter hypermethylation was investigated as a control. As per the analyses of p15 and p21 methylation, all MSP and TTGE primers and probes for p27 were designed to be located within the promoter associated CpG island and near the major transcriptional start site

(GenBank Accession Numbers AB005590 and S76986) as illustrated in Figure 4.2C.

Consistent with the finding that p27 mRNA was strongly expressed in all AML cell lines and patients, including those samples that lacked p15 and/or p21 mRNA, no p27 promoter hypermethylation in 47 of 47 samples available for analysis was observed using either MSP or TTGE (Fig. 4.5). This included all AML cell lines (n = 7) and patient samples (n = 24) analyzed by RPA as well as the 16 patient samples for which expression data was not available. Moreover, no methylation of the p27 gene was detected in the DNA of 13 non-leukemic controls. The specificity of p27 MSP, like that of p15 and p21, was confirmed by sequencing methylated PCR products generated from the universally methylated positive control and unmethylated PCR products from individual AML cases (Fig. 4.5D).

4.1.4. DISCUSSION

Most genetic alterations that have been characterized in hematopoietic malignancies concern oncogene activation, yet recently, a large body of evidence has suggested that the loss of tumor suppressor gene activity is an important event in the development of such cancers. Examples of potential tumor suppressors are the CDKI small regulatory proteins which exert a negative control on the cell cycle by inhibiting the kinase activity of CDKs. Previous studies implicating CDKI genes in human leukemogenesis (Drexler, 1998; Tsihlias et al., 1999) and the critical role epigenetic silencing plays in neoplasia (Verma and Srivastava, 2002)

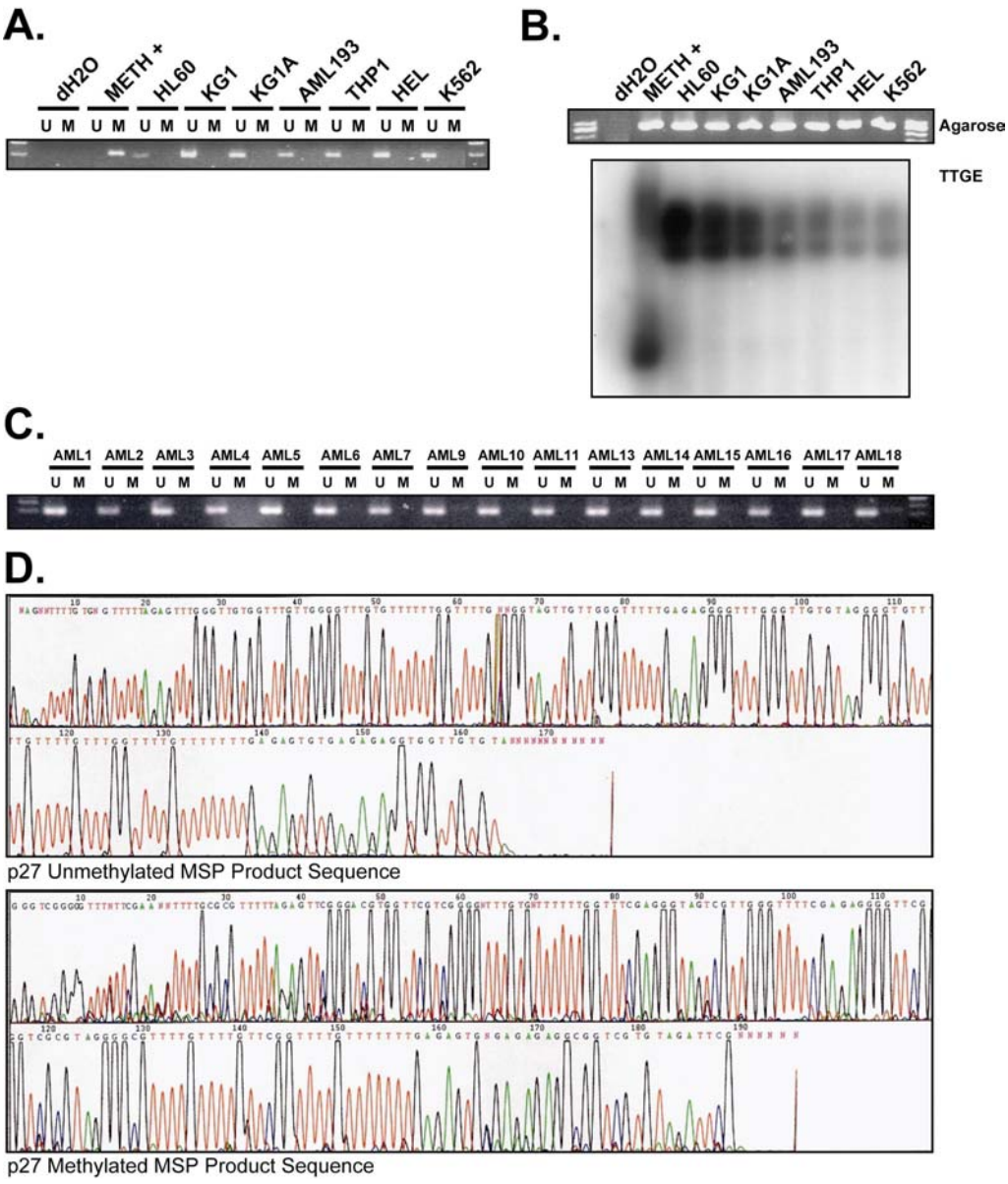

Figure 4.5 p27 Methylation in Human AML. **A)** MSP analysis of bisulfite modified AML cell line DNA. Commercially available universally methylated bisulfite modified DNA was used as a methylated positive control (METH +) and water as a negative reagent control (dH2O). **B)** TTGE analysis of 22 CpG sites within the p27 promoter in AML cell lines. Differentially methylated sequences within the p27 amplicons are resolved according to differences in melting temperature and compared to the known methylated control (METH +). **C)** MSP analysis of bisulfite modified AML patient sample DNA. **D)** Representative MSP product sequences from both the unmethylated and methylated PCR reactions. U: unmethylated MSP reaction; M: methylated MSP reaction.

prompted the investigation of mRNA levels and promoter methylation status of the p15, p21, and p27 CDKI genes in human AML.

Hypermethylation of the p15 promoter and associated transcriptional silencing has been previously reported in a host of hematological malignancies including Burkitt's lymphoma, ALL, CML, MDS and AML (Herman et al., 1997; Nguyen et al., 2000; Quesnel et al., 1998). The frequency of p15 hypermethylation presented in this study (83%) is consistent with previously reported AML data (Herman et al., 1997; Melki et al., 1999) and further implicates p15 promoter hypermethylation in the molecular pathogenesis of human AML. Importantly, p15 mRNA was absent in 79% of cell lines and patient samples and was absent in all testable cases harboring p15 promoter hypermethylation, even though unmethylated p15 alleles were often detected in the patient samples by MSP. The presence of unmethylated alleles within the patient samples harboring p15 hypermethylation could be a result of a small percentage of contaminating normal cells in the initial bone marrow or peripheral blood sample. However, if this is the case, they did not have a significant effect on p15 mRNA status. Another possibility is that p15 promoter hypermethylation in AML is monoallelic, yet this is unlikely as other studies using bisulfite sequencing have shown heterogeneous biallelic p15 promoter hypermethylation, varying greatly in methylation density and not limited to a single allele (Cameron et al., 1999b). This is consistent with the TTGE data of the present study and the denaturing gradient gel electrophoresis (DGGE) data previously published (Aggerholm et al., 1999), which reveal a high

degree of both intra- and interindividual heterogeneity of p15 promoter hypermethylation in AML patients.

As mentioned, p15 is normally upregulated by TGF-β1, possibly mediating the cell cycle arrest and reduced phosphorylation of RB seen following TGF-β1 treatment (Laiho et al., 1990). Furthermore, TGF-β1 is a potent growth inhibitor of hematopoietic stem cells and early progenitor cells, which are considered the putative cells of origin in AML. For these normal cells, TGF-β1 inhibits proliferation despite the presence of mitogenic stimuli (Hooper, 1991). Thus, ablation of p15 function, by either genetic or epigenetic means, could lead to escape from normal growth suppression. Although this attractive hypothesis has yet to be confirmed, evidence to support this notion has been offered by Dodge et al. who reported p15 protein induction and G1 arrest in HL60, but not KG1 or KG1A cells, following treatment with the differentiating agent phorbol 12-myristate 13-acetate (PMA) (Dodge et al., 2001). The authors, similar to the data presented herein, also report partial p15 hypermethylation in the KG1 cell line and complete p15 promoter hypermethylation in the KG1A cell line as detected by MSP. Furthermore, the upregulation of p15 following TGF-β1 treatment has been shown to be exerted at the level of transcription through promoter associated GC-rich Sp1 consensus sites (Li et al., 1995). As such, it is very tempting to hypothesize that p15 promoter hypermethylation in AML blocks normal p15 transcriptional induction by interfering with growth factor signaling at the promoter.

Similar to p15, a high frequency of AML samples lacked p21 mRNA (39%). However, unlike p15, the absence of p21 mRNA was not associated with promoter hypermethylation. Analysis of p21 methylation status in hematological malignancies and solid tumors have shown variable, and in some cases discrepant results (Roman-Gomez et al., 2002; Shen et al., 2002; Ying et al., 2004). A recently reported large study of primary lymphoma and carcinoma tumors and cell lines found p21 hypermethylation by MSP in only 5 of 245 (2.0%) samples (Ying et al., 2004). A summary of all pertinent literature by this group found p21 hypermethylation to be rare in tumors in general, especially when analyzed by bisulfite-based methods such as MSP (Ying et al., 2004). They also noted that both studies employing restriction enzyme based assays, which analyze the methylation status of very few CpG sites, detected methylation at high frequencies, including normal tissues in some instances (Chen et al., 2000; Roman-Gomez et al., 2002; Ying et al., 2004). This observation may be pertinent, as recently Roman-Gomez et al., reported the presence of p21 promoter hypermethylation in 51 of 124 (41%) ALL patients using a restriction enzyme based assay (Roman-Gomez et al., 2002), whereas, Shen et al., found no methylation of the p21 promoter in a group of 31 ALL patients using bisulfite-based methods (Shen et al., 2002). Consistent with the data from Shen et al., our lab recently reported the absence of p21 promoter hypermethylation in 28 T-cell lymphoblastic lymphoma/leukemia cell lines and patient samples using both MSP and TTGE assays (Scott et al., 2004).

As MSP is the technique most widely used to assess methylation status, it was employed for evaluation of the AML samples in the present study. The

absence of p21 promoter hypermethylation reported herein was confirmed by TTGE, which allows the analysis of multiple CpGs within the p21 promoter and limits the influence of primer amplification bias on the assessment of methylation status. Thus, the data presented herein is the first to analyze p21 promoter hypermethylation in AML, concurs with the analysis of ALL by Shen et al., and argues against a role for p21 promoter hypermethylation in the molecular pathogenesis of AML. However, other epigenetic mechanisms of p21 silencing independent of promoter methylation, such as histone deacetylation, can not be ruled out.

Interestingly, a restriction based study by Chen et al., found CpG methylation of a distal STAT binding site within the p21 promoter (Fig. 4.2B) to be associated with decreased constitutive p21 mRNA expression in rhabdomyosarcoma (Chen et al., 2000). Thus, while gene silencing as a consequence of methylation is most often associated with dense methylation within promoter CpG islands (Cameron et al., 1999b), future studies of distal CpG sites in AML may also be of interest.

Similar to the CIP/KIP CDKI family member p21, mutations and deletions of p27 are rare in human cancer (Ponce-Castaneda et al., 1995), prompting investigation into alternative mechanisms of p27 inactivation, including promoter hypermethylation (Go, 2003; Kibel et al., 2001; Nakamura et al., 2001; Nakatsuka et al., 2003; Worm et al., 2000). Data from malignant melanoma has suggested that DNA methylation may cause monoallelic p27 silencing (Worm et al., 2000). Given the paucity of p27 mutations or deletions, this melanoma study supports a role for

p27 haploinsufficiency in human cancer. However, melanoma cell lines harboring one methylated and one wild-type p27 allele showed reduced p27 mRNA expression. In contrast, the presented AML data reveal strong p27 mRNA expression in all samples arguing against epigenetic silencing of p27 as an important mechanism in the pathogenesis of human AML. Consistent with the expression data, no evidence of p27 hypermethylation was found in any of the 47 AML samples analyzed. The absence of p27 hypermethylation observed in AML is in contrast to studies of predominantly nodal based B-cell lymphomas and nasal cavity NK/T-cell lymphomas (Nakatsuka et al., 2003) and primary central nervous system B-cell lymphomas (Nakamura et al., 2001) but consistent with analysis of primary gastrointestinal B-cell lymphomas (Go, 2003). The differing frequencies of p27 promoter hypermethylation suggest that the importance of p27 inactivation may vary among subtypes of hematological malignancies. Alternatively, p27 disruption in those hematological malignancies lacking promoter methylation may occur by other mechanisms, for example increased proteasome mediated protein degradation (Loda et al., 1997).

In summary, the results indicate that p15 and p21 mRNA is absent in human AML cell lines and patient material at a high frequency, whereas, p27 mRNA levels are not reduced. Thus, these findings implicate the ablation of certain TGF-β1 pathway CDKI genes (p15 and p21) but not others (p27) in the pathogenesis of this disease. The findings that p15 mRNA absence is frequently accompanied by promoter hypermethylation in AML, whereas, reduced p21 expression occurs independently of promoter hypermethylation are consistent with

the literature and suggest that within the same tumor, epigenetic silencing of CDKI genes may involve methylation dependent or independent mechanisms depending on the target gene.

4.2. DEMETHYLATION AND RE-EXPRESSION OF p15 BY 5-Aza-dC IS ASSOCIATED WITH H3 MODIFICATIONS AND RELEASE OF MeCP2 AND SUV39h1 AT THE PROMOTER

4.2.1. 5-Aza-dC Demethylates and Induces p15 in AML193 Cells

As mentioned, p15 promoter hypermethylation and associated transcriptional silencing has been reported in various hematological malignancies (Herman et al., 1997; Melki et al., 1999; Nguyen et al., 2000; Quesnel et al., 1998; Scott et al., 2004), and is consistent with our previous investigation of CDKIs in human AML. While p15 promoter hypermethylation occurs frequently in AML, little is known about the components of repressor complexes that effect epigenetic silencing of densely methylated p15 promoter alleles or their relationship to covalent histone modifications. Moreover, 5-Aza-dC has recently been shown to have both methylation dependent and independent activities depending on the cell type and methylation status of the target gene (Schmelz et al., 2005). Given the identification of AML cell lines harboring complete p15 promoter hypermethylation in the previous study, AML193 cells were used in combination with the DNMT inhibitor 5-Aza-dC (Fig. 1.8) to confirm the involvement of CpG island hypermethylation in p15 transcriptional silencing and to investigate the mechanism of p15 gene silencing in human AML.

To determine if 5-Aza-dC can demethylate the p15 promoter and reconstitute p15 mRNA in AML193 cells, a time course experiment was designed with 4 μM 5-Aza-dC treated over a period of four days with both DNA and RNA

harvested every 12 hours (Fig. 4.6). A dose response experiment was initially done to determine optimal 5-Aza-dC dosage (data not shown), which was consistent with previous studies that have reported robust DNA demethylation with acceptable toxicity in the low micromolar dose range (Covey and Zaharko, 1984; Davis et al., 1989; Issa, 2003; Issa et al., 2004; Momparler and Laliberte, 1990). As expected, at the zero time point the p15 promoter was completely methylated (Fig. 4.6A) and mRNA was undetectable (Fig. 4.6B) as determined by TTGE and RTPCR, respectively. However, after approximately 60 hours of 5-Aza-dC treatment, unmethylated p15 alleles were detectable by TTGE in association with detectable mRNA (Fig. 4.6). Importantly, no change in p15 methylation status or mRNA was detected following treatment with a PBS vehicle control throughout the same time period and as a control for cDNA integrity and equivalent RTPCR template quantity, the housekeeping gene β-Actin was amplified in all time point samples. This data provides further evidence for the central role that aberrant promoter hypermethylation plays in p15 gene silencing and is consistent with previous reports that used 5-Aza-dC to induce p15 mRNA in KG1A cells (Herman et al., 1996b), and those that used 5-Aza-dC to demethylate and reactivate the neighboring p16 gene in colon cancer cell lines (Herman et al., 1995).

4.2.2. 5-Aza-dC Mediated Induction of p15 is Associated with MeCP2 and SUV39h1 Release at the Promoter

A direct relationship between DNA hypermethylation mediated transcriptional silencing and the modification of regional chromatin was established

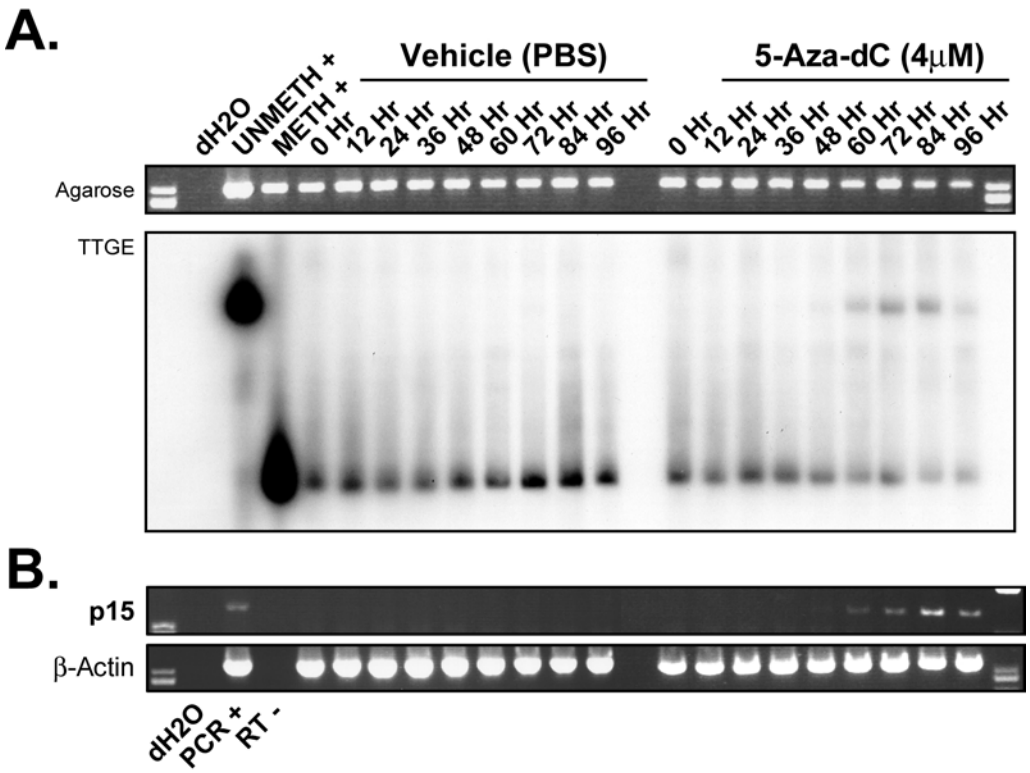

Figure 4.6 Demethylation and Re-expression of p15 by 5-Aza-dC Treatment in AML193 Cells. A) TTGE analysis of bisulfite modified DNA from a 5-Aza-dC time course assay in AML193 cells. DNA was harvested every 12 hours for four days from cells treated with either a vehicle control or 5-Aza-dC. Differentially methylated sequences within the p15 amplicons were resolved by TTGE according to differences in melting temperature and compared to the migration of controls (UNMETH + and METH +). Water was used as a PCR reagent control (dH2O). **B)** RTPCR analysis of the p15 gene using RNA harvested from the same 5-Aza-dC time course assay described in **(A)**. Included in each amplification is a PCR positive control (PCR +), and water and reverse transcriptase negative controls (dH2O and RT -). The housekeeping gene β-Actin was amplified in all cell lines to ensure RNA integrity and equivalent cDNA template quantity between samples. Note the presence of detectable p15 mRNA by RTPCR in concordance with promoter demethylation by TTGE following 60 to 72 hours of 5-Aza-dC treatment.

in 1998 by the observation that methylated DNA associates with a repressive protein assembly that includes members of the MBD family and HDAC activity (Jones et al., 1998). As mentioned, since then MBD proteins have been determined by ChIP assays to be physically associated with hypermethylated promoters in hepatocellular carcinoma (Bakker et al., 2002), colon cancer (Magdinier and Wolffe, 2001), bladder cancer (Nguyen et al., 2002), and T-cell leukemia cell lines (El-Osta et al., 2002). Moreover, the MBD MeCP2 has recently been shown to not only colocalize with HDAC activity, but also that of H3 lysine 9 methyltransferase activity (Fuks et al., 2003b), implying that MeCP2 acts as a bridge between DNA methylation and chromatin remodeling. As such, in order to examine if MBDs, HMTs, and HDACs are present at the hypermethylated p15 promoter in AML193 cells, ChIP assays were employed with antibodies specific for MeCP2, SUV39h1 and HDAC1, and PCR primers specific for the p15 promoter region (Fig. 4.2).

Similar to the methylated p16 promoter reported in bladder cancer (Nguyen et al., 2002) and the methylated MDR1 promoter in T-cell leukemia (El-Osta et al., 2002), MeCP2 was found to be associated with the hypermethylated p15 promoter region in AML193 cells and was reduced by almost 9 fold following treatment with 4 μM 5-Aza-dC (Fig. 4.7A), a dosage consistent with p15 promoter demethylation and re-expression of the gene. Along with MeCP2, the p15 promoter region also harbored the H3 lysine 9 specific methyltransferase SUV39h1, which was reduced 4 fold following 5-Aza-dC treatment (Fig. 4.7B). However, a ChIP assay for HDAC1 was unable to immunoprecipitate p15 promoter region DNA fragments in the absence or presence of 5-Aza-dC (Fig. 4.7C). Although repressive complexes

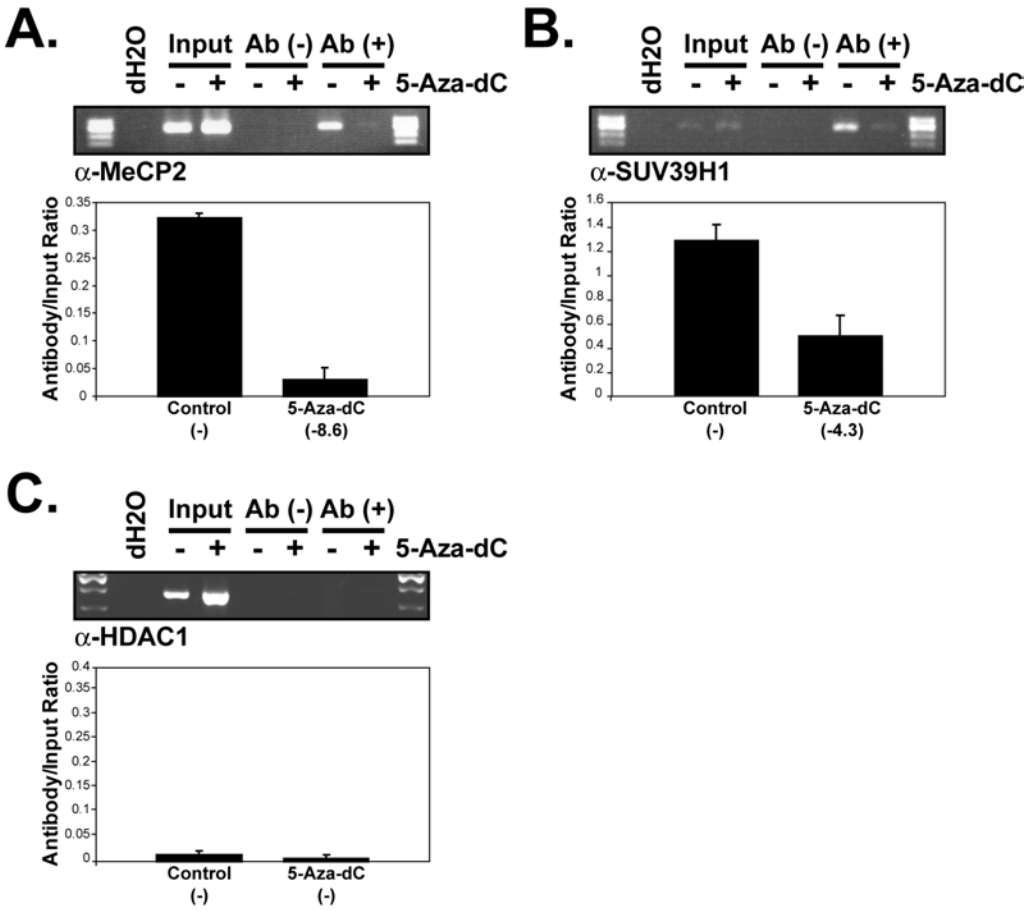

Figure 4.7 Epigenetic Proteins at the p15 Promoter in AML193 Cells. Illustrated is the ethidium bromide staining of ChIP PCR products from input positive controls (Input), no-antibody negative controls [Ab (-)], and specific antibody immunoprecipitates [Ab (+)]. Each experiment was performed in the absence and presence of 5-Aza-dC and included in each PCR is a water negative reagent control (dH2O). Lower panels graphically represent semi-quantitation of the ChIP assay using densitometry of the PCR products from immunoprecipitated samples normalized to their input counterparts and expressed numerically in brackets as fold change following 5-Aza-dC treatment. ChIP was performed with antibodies for MeCP2 [α-MeCP2; **(A)**], SUV39h1 [α-SUV39h1; **(B)**], and HDAC1 [α-HDAC1; **(C)**].

that contain MeCP2 are known to harbor HDAC activity it is important to note that to date, many members of the mammalian HDAC superfamily have been identified and therefore other HDACs beyond that of HDAC1 could be involved at the hypermethylated p15 locus. Future ChIP assays are required to define exactly which HDAC, if any, is present at the p15 promoter.

4.2.3. 5-Aza-dC Mediated Induction of p15 is Associated with Changes in H3 Acetylation

Although HDAC1 was not found to be associated with the p15 promoter in AML193 cells, hypermethylated CpG islands are typically associated with an inaccessible chromatin conformation and deacetylated histones (Bachman et al., 2003; Nguyen et al., 2001). Increased acetylation of histones by pharmacological inhibition of HDACs causes an increase in DNA accessibility through chromatin remodeling (Gui et al., 2004; Richon et al., 2000), which presumably assists transcription by allowing the RNA polymerase machinery access to important enhancer elements and transcriptional start sites. Interestingly, evidence also exists supporting a role for 5-Aza-dC induced enrichment of histone acetylation at promoter regions previously silenced by CpG island hypermethylation (Coombes et al., 2003; Fahrner et al., 2002; Nguyen et al., 2002). Given the induction of p15 mRNA and release of repressive components at the p15 promoter following 5-Aza-dC treatment and the previous studies looking at 5-Aza-dC induced histone acetylation in other genes and cell types, ChIP assays were employed to test if H3 acetylation is affected following demethylation of the p15 promoter in AML193

cells. If MeCP2 recruits HDAC activity to aid in the silencing of p15 in AML193 cells, then diminished levels of histone acetylation should be associated with this gene. Indeed, H3 acetylation at lysine residues 9 and 14 was found to be absent at the hypermethylated p15 promoter region in AML193 cells, yet was enriched by approximately 16 fold following treatment with 4 μM 5-Aza-dC (Fig. 4.8A). To examine acetylation at additional sites of H3, ChIP assays were performed with antibodies specific for single H3 acetylated lysine residues 18 and 23. An increase in H3 acetylation was observed at the lysine 18 residue, where 5-Aza-dC treatment caused a 2 fold enrichment (Fig. 4.8B), however the ChIP assay for acetylated H3 lysine 23 was unable to immunoprecipitate p15 promoter region DNA fragments in the absence or presence of 5-Aza-dC (Fig. 4.8C). Taken together, similar to the neighboring p16 gene in bladder cancer (Nguyen et al., 2002) and head and neck squamous cell carcinoma (HNSCC) (Coombes et al., 2003), 5-Aza-dC induced demethylation and re-expression of the p15 gene in AML193 cells is associated with a regional enrichment of H3 acetylation, in this case specifically at lysine residues 9, 14, and 18.

4.2.4. 5-Aza-dC Mediated Induction of p15 is Associated with Changes in H3 Methylation

Unlike histone acetylation, regional histone methylation is not always associated with either a strictly active or inactive transcriptional state. In fact, as mentioned previously, methylation of specific histone amino acids correlates with differing states of transcriptional activity depending on which lysine or arginine

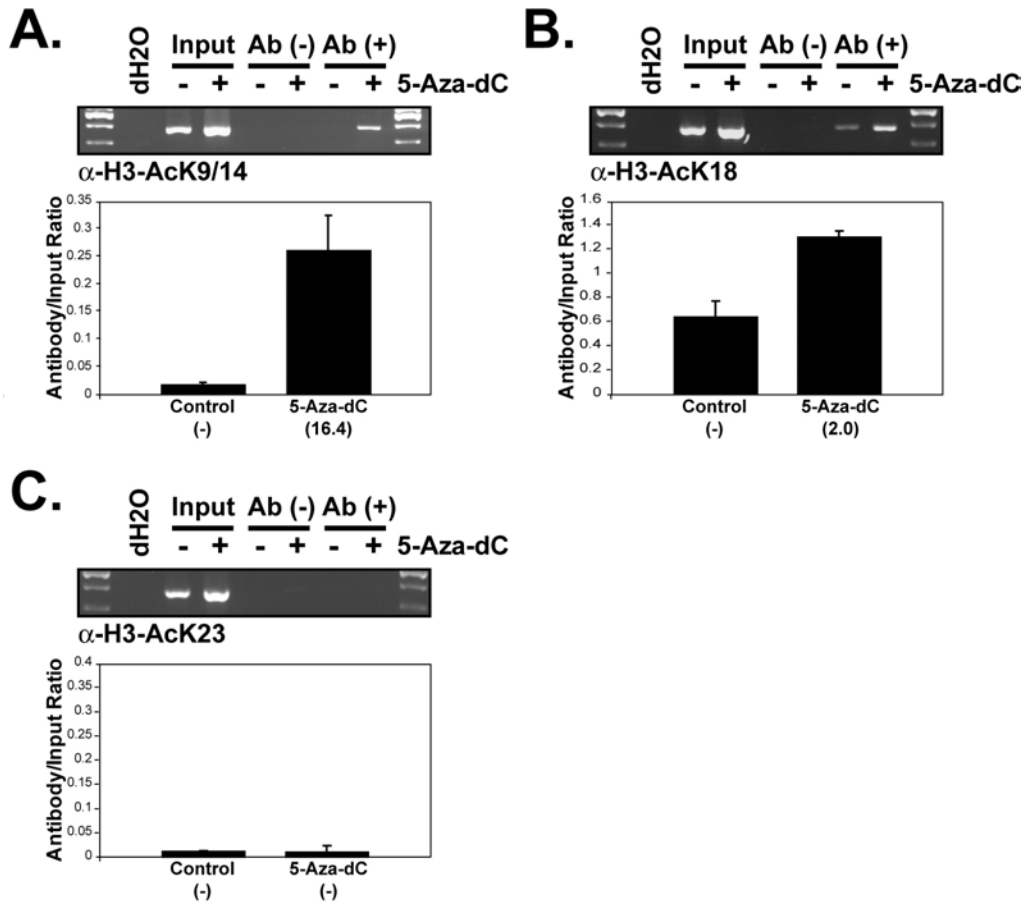

Figure 4.8 H3 Acetylation at the p15 Promoter in AML193 Cells. Illustrated are ChIP experiments as described in Figure 4.7 with antibodies for acetylated H3 lysine 9 and 14 [α-H3-AcK9/14; **(A)**], acetylated H3 lysine 18 [α-H3-AcK18; **(B)**], and acetylated H3 lysine 23 [α-H3-AcK23; **(C)**].

residue is modified (Lachner and Jenuwein, 2002). The best characterized are those of H3 lysine 4 and 9, where methylation of lysine 4 consistently correlates with transcriptionally active chromatin and methylation of lysine 9 consistently correlates with transcriptionally inactive chromatin (Kondo et al., 2003). Given the association between H3 lysine 4 methylation and transcriptionally active chromatin regions in higher eukaryotes (Schneider et al., 2004; Strahl et al., 1999), one would expect this modification to be enriched at the p15 gene in AML193 cells following 5-Aza-dC treatment. In contrast, H3 lysine 9 methylation is reported to be involved in chromatin compaction through a repressive complex that associates with DNA hypermethylation. Interestingly, it has recently been shown that DNMT1 and DNMT3a associate with the H3 lysine 9 specific methyltransferase SUV39h1 (Fuks et al., 2003a), which is most likely linked by MeCP2 (Fuks et al., 2003b), substantiating the connection between DNA and histone methylation. Given our previous data showing occupation of the p15 promoter region by both MeCP2 and SUV39h1 in AML193 cells, one would expect H3 lysine 9 methylation to be enriched at this locus prior to 5-Aza-dC treatment. Thus, to test for the presence or absence of H3 lysine 4 and 9 methylation at the hypermethylated p15 promoter region, ChIP assays were employed in the absence and presence of 5-Aza-dC in AML193 cells. To examine the methylation status of arginine H3 residues, ChIP assays were performed with an antibody specific to H3 dimethylated arginine 17.

As shown in Figure 4.9A, demethylation and re-expression of p15 by 5-Aza-dC resulted in an almost 5 fold enrichment of dimethylated H3 lysine 4 at the p15 promoter region, which is consistent with the hypothesis that H3 lysine 4

methylation plays a role in transcriptionally active chromatin. In contrast, the p15 promoter region was associated with dimethylated H3 lysine 9 at high levels prior to drug administration yet was reduced by almost 3 fold following treatment with 5-Aza-dC (Fig. 4.9B), suggesting an inverse correlation between the methylation of lysines 4 and 9 on H3. Similar to the MeCP2 and H3 acetylation data, these findings are consistent with aforementioned reports on the p16 gene in bladder cancer (Nguyen et al., 2002) and HNSCC (Coombes et al., 2003).

Acetylation of H3, a modification traditionally associated with transcriptionally active chromatin, has recently been shown to tether the aforementioned arginine HMT CARM1 to the H3 amino-terminal *in vivo* (Daujat et al., 2002) and methylation of H3 arginine 17, the major target of CARM1 methylation, has been shown to be associated with gene activation (Bauer et al., 2002). Hence, it stands to reason that induction of the p15 gene in AML193 cells by 5-Aza-dC would be marked by H3 arginine 17 methylation enrichment, and as shown in Figure 4.9C, 5-Aza-dC induces this modification almost 4 fold. To our knowledge, this is the first study to describe an inverse correlation between DNA hypermethylation and H3 arginine methylation.

4.2.5. 5-Aza-dC Mediated Changes in H3 Modifications are Gene Specific

It is important to note that the H3 modification observations from the previous ChIP assays could be the result of global changes in the cellular levels of modified H3. To confirm that the changes observed by ChIP analysis before and

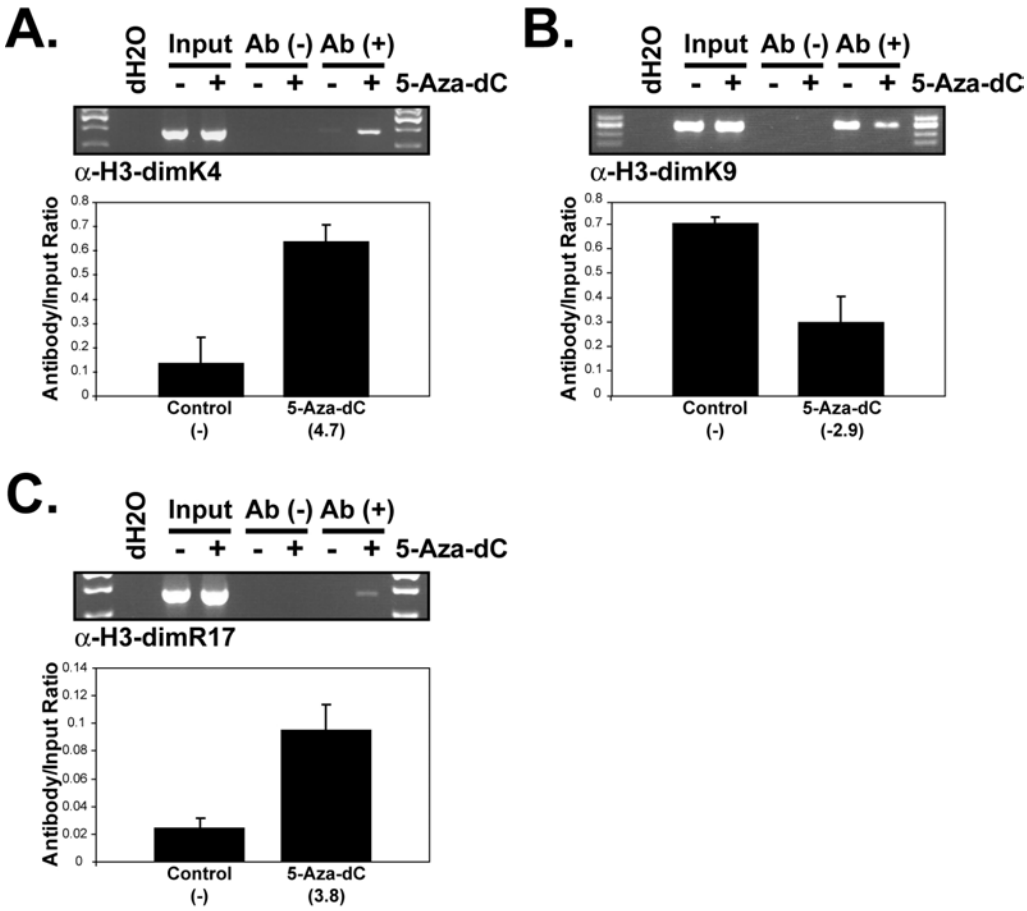

Figure 4.9 H3 Methylation at the p15 Promoter in AML193 Cells. Illustrated are ChIP experiments as described in Figure 4.7 with antibodies for dimethylated H3 lysine 4 [α-H3-dimK4; **(A)**], dimethylated H3 lysine 9 [α-H3-dimK9;**(B)**], and dimethylated H3 arginine 17 [α-H3-dimR17; **(C)**].

after 5-Aza-dC treatment are gene specific observations, western blots were performed to measure the total protein levels of modified H3.

Figure 4.10 shows that the overall levels of H3 acetylation (lysines 9/14, 18, and 23) and H3 methylation (lysines 4 and 9, and arginine 17) do not dramatically change after 5-Aza-dC treatment of AML193 cells (Fig. 4.10), suggesting that the chromatin remodeling detected by ChIP analysis at the p15 promoter region are gene specific events rather than a reflection of global changes in histone modification. The experiment was performed on two types of total protein, one which was extracted by standard methods, and another which was extracted using a protocol that enriches for the histone fraction. In both cases, no major changes in histone protein level were observed when compared to the levels of total unmodified H3.

4.2.6. 5-Aza-dC and TSA Combinatorial Treatment Synergistically Induces p15 in AML193 Cells

As mentioned, previous reports have shown that combinatorial inhibition of both DNMT and HDAC activities can synergistically reactivate epigenetically silenced genes *in vitro* (Cameron et al., 1999a) and the potential for combinatorial treatment as a cancer therapy is currently being evaluated (Shaker et al., 2003; Zhu and Otterson, 2003). Therefore, to test for synergistic induction of p15 by pharmacologic inhibition of DNMT and HDAC in AML193 cells, RTPCR was employed following treatment with both 5-Aza-dC and the well known HDAC inhibitor TSA. As shown in Figure 4.11A, treatment of AML193 cells with 5-Aza-

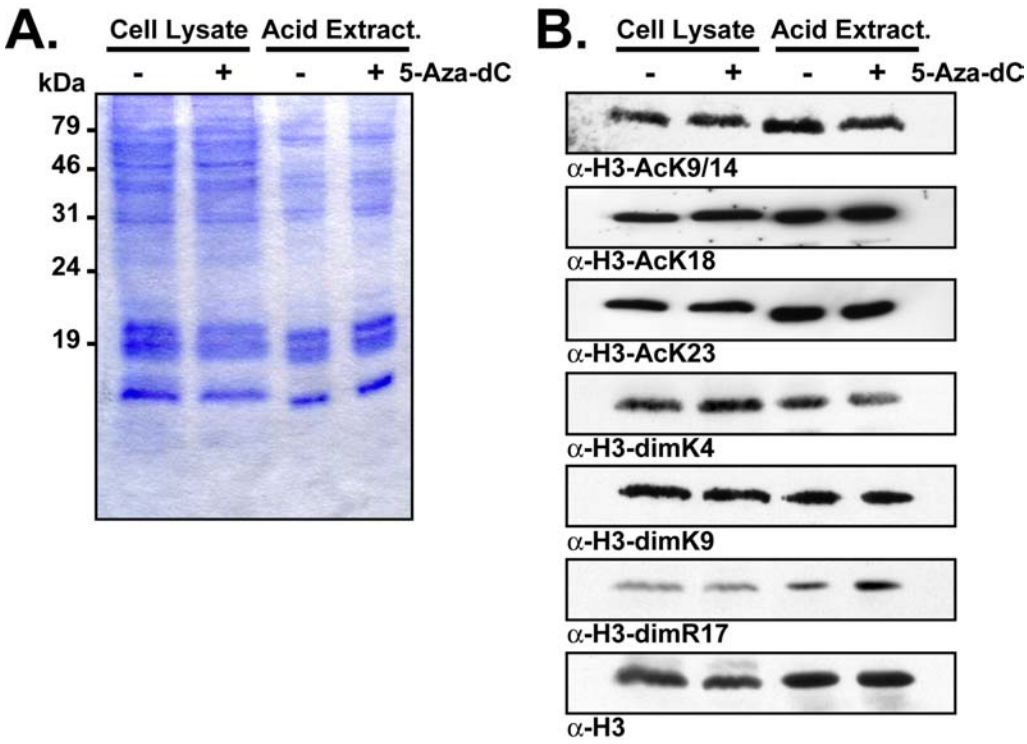

Figure 4.10 Effects of 5-Aza-dC on Global H3 Modifications in AML193 Cells. A) Coomassie stained SDS-PAGE gel showing resolution of total cellular proteins. **B)** Western blot analysis of modified histones with antibodies used for the previous ChIP assays and an antibody for total unmodified H3 (α-H3). Protein was extracted using two separate protocols as described in the *Materials and Methods*; a standard cell lysate protocol (Cell Lysate) and a protocol that enriches for the histone fraction (Acid Extract.), both in the absence and presence of 5-Aza-dC.

dC, similar to the experiment in Figure 4.6, resulted in re-expression of detectable p15 mRNA. Inhibition of HDAC activity by treatment of AML193 cells with TSA alone for 12 hours had no effect on p15 induction, implying that DNA hypermethylation is a dominant process over histone deacetylation at this locus. However, treatment of AML193 cells with TSA for the final 12 hours of a three day 5-Aza-dC treatment synergistically induced detectable p15 mRNA. This pattern of mRNA induction has been shown in other systems following DNMT and/or HDAC inhibition and is emblematic of a gene which is silenced by promoter hypermethylation. For example, similar mRNA induction patterns have been reported for the MLH1, TIMP3 and p16 genes in colon cancer (Cameron et al., 1999a) and the ERα gene in breast cancer (Yang et al., 2001), and the results suggest that for genes silenced by promoter hypermethylation, DNA demethylation is a prerequisite to induce transcription by inhibiting HDAC activity.

To assess the levels of histone acetylation at the p15 promoter following treatment with 5-Aza-dC and/or TSA, ChIP assays were employed using α-H3-AcK9/14 (Fig. 4.11B). Similar to the previous experiment illustrated in Figure 4.8, 5-Aza-dC induced H3 acetylation in concert with re-expression of p15 mRNA. In contrast, treatment with TSA alone was unable to induce regional H3 acetylation, reflecting its inability to induce transcription of this gene (Fig. 4.11A). Yet, when TSA was treated in combination with 5-Aza-dC, synergistic induction of p15 mRNA was observed in association with an increase in H3 acetylation at the p15 promoter region. Although a synergistic enrichment of H3 acetylation was not observed following combinatorial treatment, a trend towards a greater induction of

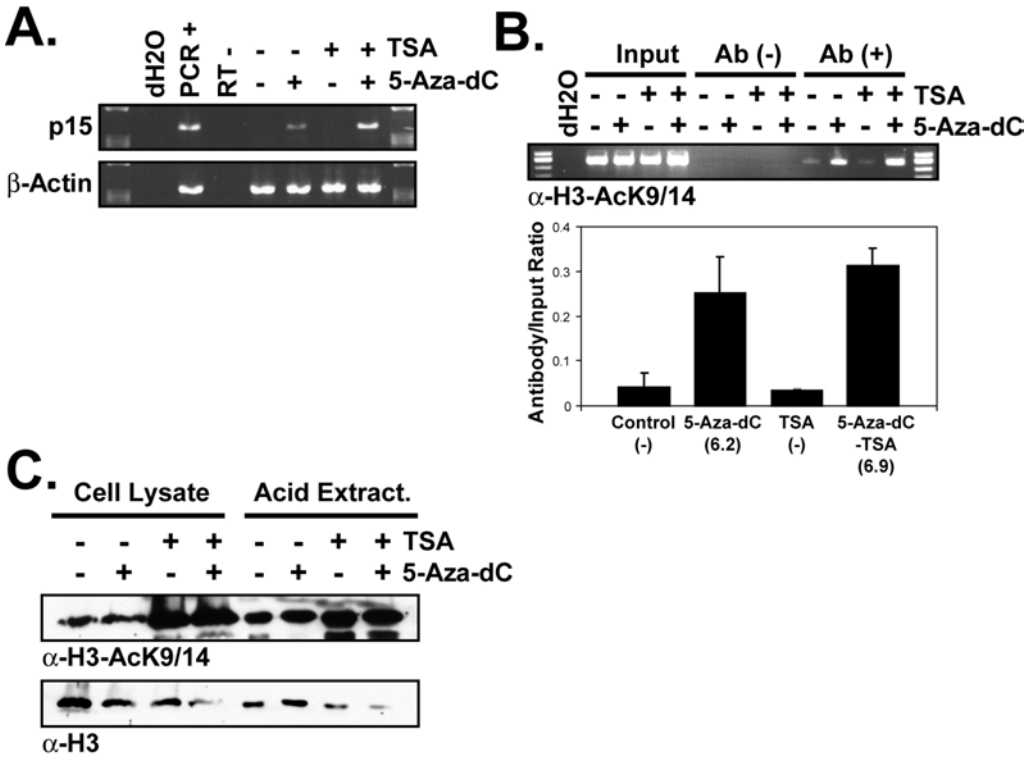

Figure 4.11 Effects of 5-Aza-dC and TSA Combinatorial Treatment in AML193 Cells. A) p15 RTPCR following treatment with vehicle controls, 5-Aza-dC, TSA, and combinatorial treatment with 5-Aza-dC and TSA. Included in each RTPCR amplification is a PCR positive control (PCR +) and water and reverse transcriptase negative controls (dH2O and RT -). The housekeeping gene β-Actin is amplified in all samples to ensure RNA integrity and equivalent cDNA template quantity between samples. **B)** Illustrated is a ChIP experiment as described in Figure 4.7 immunoprecipitated with α-H3-AcK9/14 and tested following the same treatment conditions as in **(A)**. **C)** Western blot analysis using α-H3-AcK9/14 and α-H3 on protein lysates harvested as described in 4.10, following the same treatment conditions as in **(A)**.

H3 acetylation was consistently detected in AML193 cells. To test the effect of 5-Aza-dC and/or TSA treatment on global cellular levels of acetylated H3 in AML193 cells, western blots were performed utilizing α-H3-AcK9/14 on cell lysates and enriched histone protein fractions (Fig. 4.11C). Similar to the experiment illustrated in Figure 4.10, 5-Aza-dC failed to significantly alter the levels of acetylated H3 when treated alone, however TSA – a well known inhibitor of HDAC activity – greatly induced global acetylation of H3 when treated as a single agent and when treated in combination with 5-Aza-dC. This is of interest as even though global acetylation is induced by TSA, the ChIP data reveals that the p15 promoter retains deacetylated histones following TSA treatment. This again suggests that DNA methylation preserves DNA:histone complexes in a transcriptionally inert conformation and that hypermethylated genes require promoter demethylation prior to pharmacologic HDAC inhibition to induce transcription.

4.2.7. DISCUSSION

Taken together, the 5-Aza-dC time course experiment illustrated in Figure 4.6 and the ChIP data summarized in Figure 4.12 demonstrate that the p15 gene is in a transcriptionally inactive state when its promoter associated CpG island is hypermethylated in AML193 cells. Histones, the key protein components of chromatin, located near the p15 transcriptional start site and within the CpG island are marked by H3 amino-terminal deacetylation, enriched lysine 9 methylation, diminished lysine 4 and arginine 17 methylation and presumably an inaccessible

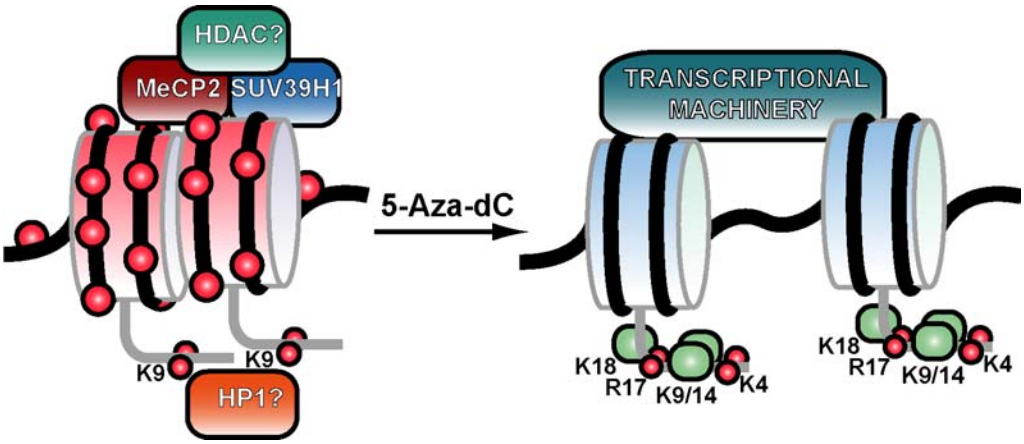

Figure 4.12 Summary of ChIP Data Before and After 5-Aza-dC Treatment. Illustrated is a hypothetical schematic representation of the p15 promoter region in AML193 cells before and after treatment with 5-Aza-dC. DNA: black lines; histone octamers: cylinders; protruding histone amino tails: gray lines; methyl moieties: red circles; acetyl moieties: green rectangles.

chromatin conformation. Furthermore, p15 demethylation by 5-Aza-dC reverses local covalent histone modifications without altering their global levels and results in p15 induction. Global HDAC inhibition by TSA treatment is insufficient to reactivate methylated p15 alleles and requires 5-Aza-dC pretreatment in order to synergistically enhance p15 mRNA production. This data corroborates previous reports on both p15 (Cameron et al., 1999a) and p16 (Coombes et al., 2003; Kondo et al., 2003; Nguyen et al., 2002) induction and supports the hypothesis that promoter hypermethylation is the dominant epigenetic process over histone deacetylation. Similar to the experiments with DNMT and HDAC inhibitors, it would be interesting to study the effects of a pharmacologic inhibitor of histone methylation, specifically H3 lysine 9 methylation; however such a compound does not yet exist.

MeCP2 is a chromosomal protein that preferentially binds to DNA that contains a single symmetrically methylated CpG (Nan et al., 1996) and has been shown in several solid tumor cell lines to localize to hypermethylated tumor suppressor gene promoters (Nguyen et al., 2001; Nguyen et al., 2002). Our data involving the hypermethylated p15 gene supports this concept and extends the current body of MeCP2 literature to include a role for this MBD in hematologic malignancy tumor suppressor gene silencing. The co-localization of SUV39h1 at this same region is also consistent with the literature as MeCP2 has recently been shown to harbor a protein complex containing H3 lysine 9 methyltransferase activity (Fuks et al., 2003b). As SUV39h1 is an H3 lysine 9 specific HMT, our data would suggest that the HMT activity observed in the MeCP2 complex reported

by Fuks et al. would be that of SUV39h1, yet it should be noted that other mammalian lysine 9 specific HMTs do exist.

As previously mentioned, methylated H3 lysine 9 is consistently associated with silent heterochromatic regions yet the exact role this modification plays in this epigenetic process is not fully understood. The prevailing dogma involves the heterochromatic adaptor protein HP1, which has been implicated in both gene silencing and supra-nucleosomal chromatin structure. Methylated H3 lysine 9 is a binding site for the chromodomain of HP1 (Bannister et al., 2001; Lachner et al., 2001), which is known to mediate gene silencing and repress transcription (Li et al., 2002a). Furthermore, HP1 and SUV39h1 have been shown to directly interact (Yamamoto and Sonoda, 2003), as has SUV39h1 and the DNMTs (Fuks et al., 2003a), which supports the hypothesis that the epigenetic machinery located at hypermethylated promoters propagates a self-reinforcing transcriptionally inert heterochromatic subdomain. Further ChIP experiments using antibodies for the three human HP1 isoforms are necessary to confirm the presence or absence of this epigenetic regulator at the hypermethylated p15 promoter region in AML cells.

The ability of 5-Aza-dC to reduce H3 lysine 9 methylation at tumor suppressor gene loci is interesting and warrants further investigation as the mechanism behind this is not known. It is possible that cytosine methylation is required to maintain histone methylation, but given the vast potential methylation marks within the histone amino-terminals, it is difficult to envision 5-Aza-dC having the ability to specifically downregulate the methylation of certain amino acids (such as H3 lysine 9) and upregulate or not effect others. Given the fact that

DNMTs have recently been shown to associate with a complex that includes HDAC activity and SUV39h1 (Fuks et al., 2003a; Vaute et al., 2002), it is tempting to hypothesize that DNMT depletion induced by 5-Aza-dC leads to H3 lysine 9 demethylation via disruption of the epigenetic silencing complex. In the same manner, this disruption could also facilitate the local enrichment of acetylated histones which is supported by the data presented herein and by studies on the hypermethylated p16 gene (Coombes et al., 2003; Kondo et al., 2003; Nguyen et al., 2002) and MLH1 gene (Fahrner et al., 2002) in solid tumor cell lines. However, this hypothesis is complicated by the fact that 5-Aza-dC treatment has also been shown to augment the expression and chromatin remodeling of a small number of unmethylated genes *in vitro* (Coombes et al., 2003; Lavelle et al., 2003; Nguyen et al., 2002), suggesting that it may have effects which are independent of cytosine demethylation.

In contrast to H3 lysine 9 methylation, H3 lysine 4 methylation was associated with a transcriptionally active p15 gene in AML193 cells. Although this finding is consistent with the literature (Santos-Rosa et al., 2002; Strahl et al., 1999), the exact function that this epigenetic mark has with respect to the transcriptional machinery is largely unknown. One possibility could arise from the fact that H3 lysine 4 methylation has recently been shown to block the binding of the aforementioned NuRD transcriptional repressor complex, which normally binds H3 amino-terminals facilitating repressive chromatin remodeling (Zegerman et al., 2002). Moreover, the authors of this study report that the H3 lysine 4 modification

is unique as methylation at H3 lysine 9 failed to prevent NuRD binding to the H3 amino-terminal, and therefore failed to inhibit NuRD mediated silencing.

Similarly, methylation of arginine residues has been shown to be associated with transcriptionally active chromatin which is supported by our finding of enriched H3 arginine 17 methylation at the p15 promoter following 5-Aza-dC treatment. Although proteins that harbor domains which specifically bind such a modification have yet to be defined, evidence does exist supporting a role for the arginine specific HMT CARM1 in transcriptional activation (Bauer et al., 2002) and histone acetylation (Daujat et al., 2002). Furthermore, deimination of the H3 amino-terminal, the process of converting histone arginine to citrulline and therefore inhibiting arginine methylation, has recently been reported to antagonize transcriptional induction of the pS2 estrogen-regulated promoter (Cuthbert et al., 2004), further supporting an *in vivo* role for arginine methylation in transcriptional activation.

Epigenetic mechanisms involving promoter hypermethylation and covalent histone modifications are clearly linked but many questions involving gene silencing remain unanswered. For example, how do cellular mechanisms target certain CpG islands for hypermethylation while others remain unaffected or is it merely a stochastic process? One possible explanation is the recently discovered ability of short interfering RNAs to induce DNA methylation and gene silencing in human cells (Kawasaki and Taira, 2004; Morris et al., 2004). Although known for some time in plants, the recent identification of this process in mammalian cells is of great interest as it provides a rationale as to how specific tumor suppressor genes

are targeted for DNA and H3 lysine 9 methylation and others are not. Furthermore, does DNA methylation direct histone modification or is the reverse true? As mentioned, evidence in *Neurospora crassa* exists supporting the notion that histone methylation directs DNA methylation to specific genomic regions (Tamaru and Selker, 2001), but the data presented herein suggests that the process is more complicated in mammalian cells and that most likely DNA methylation is the dominant process.

Drugs that affect the epigenetic machinery, including HDAC and DNMT inhibitors, are becoming increasingly more common as cancer therapeutics, particularly in leukemias. Given their inherent lack of specificity on not only cell type but target gene of interest, it is important to better understand their actions and consequences at a molecular level and to identify pharmacologic agents with acceptable toxicity profiles. Microarray analysis has revealed the significant effect that 5-Aza-dC and TSA have on gene expression in various solid tumor cell types (Suzuki et al., 2002; Yamashita et al., 2002), yet the correlation of gene expression with chromatin conformation and epigenetic modifiers are only beginning to be defined. Further ChIP assays would be useful to assist in the identification of other histone modifications and chromatin remodeling enzymes that are associated with hypermethylated p15 promoter alleles. Thus, understanding how 5-Aza-dC influences these covalent modifications in connection with transcriptional activation and chromatin accessibility will yield interesting insights into how these events become altered in disease states and perhaps how they can be addressed in therapy.

4.3. ZEBULARINE INHIBITS HUMAN AML GROWTH AND p15 PROMOTER HYPERMETHYLATION

4.3.1. Zebularine Induces and Demethylates p15 in AML193 Cells

Along with 5-Aza and 5-Aza-dC, other pharmacologic inhibitors of cytosine methylation exist, including but not limited to 5-fluoro-cytosine (Santi et al., 1983), 5,6-dihydro-5-azacytidine (Sheikhnejad et al., 1999), 4'-thio-2'-deoxycytidine (Kumar et al., 1997), and procaine (Villar-Garea et al., 2003). Although many have proven useful *in vitro*, their utility as *in vivo* anti-cancer therapeutics has been inhibited by toxicity and chemical stability concerns. The cytidine analog zebularine, originally developed as an inhibitor of cytidine deaminase (Driscoll et al., 1991; Kim et al., 1986), has recently demonstrated *in vitro* and *in vivo* DNMT inhibition and stable antitumor properties (Cheng et al., 2003; Cheng et al., 2004a; Cheng et al., 2004b). Given the previous studies investigating 5-Aza-dC mediated DNMT inhibition, the recently reported stability and minimal toxicity of zebularine, and the large collection of data involving p15 promoter hypermethylation in human leukemia, we evaluated the DNA demethylation and anti-proliferative effects of zebularine on the methylated p15 cell line AML193.

Previous studies using zebularine have reported that higher doses, when compared to common 5-Aza-dC treatments, are required for efficient cytosine demethylation and reconstitution of mRNA expression (Cheng et al., 2003; Cheng et al., 2004a). Thus, a dose response experiment encompassing 2 to 500 μM

zebularine was performed on AML193 cells followed by p15 RTPCR. As shown in Figure 4.13A, p15 mRNA is detectable following 100 and 500 μM zebularine treatment when compared to the 5-Aza-dC treated and HL60 positive controls. To confirm that the zebularine mediated induction of p15 mRNA was associated with promoter demethylation, MSP of the p15 promoter region was performed. As shown in Figure 4.13B, the vehicle control confirms complete hypermethylation of the p15 promoter in AML193 cells when compared to the unmethylated control cell line HL60 and the methylated positive control DNA. Interestingly, treatment with zebularine resulted in minor p15 promoter demethylation at dosages in the 10 to 50 μM range and substantial demethylation at 100 and 500 μM, yet only the higher doses were associated with detectable p15 mRNA. Taken together, the data suggests that higher doses of zebularine, when compared to 5-Aza-dC, are required to demethylate p15 promoter alleles *in vitro* and induce expression in AML193 cells.

As mentioned, previous reports have shown that combinatorial inhibition of both DNMT and HDAC activity can synergistically reactivate methylated genes (Cameron et al., 1999a). To determine if zebularine, like 5-Aza-dC, can also be effective in conjunction with HDAC inhibitors, AML193 cells were treated with zebularine and TSA and assayed for p15 mRNA and promoter hypermethylation (Fig. 4.13C and D). Treatment of AML193 cells with TSA for the final 12 hours of a three day zebularine treatment synergistically induced detectable p15 mRNA by 7.1 fold when compared to the vehicle control. As expected, all conditions that included zebularine induced demethylation of the p15 promoter as detected by

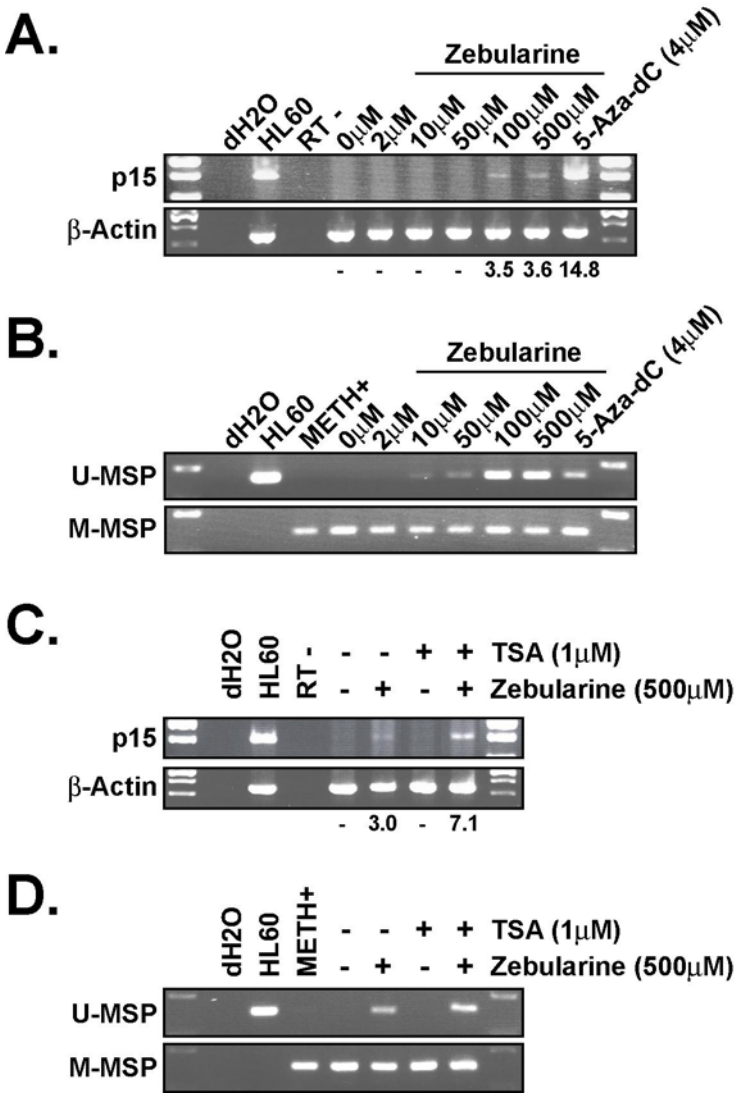

Figure 4.13 Zebularine Treatment in AML193 Cells. **A)** p15 RTPCR following a zebularine dose response. **B)** p15 MSP of the zebularine dose response shown in **(A)**. **C)** p15 RTPCR following combinatorial drug treatments as labeled. **D)** p15 MSP of the combinatorial drug treatments shown in **(C)**. Included in each RTPCR amplification [**(A)** and **(C)**] is a PCR positive control (HL60) and water and reverse transcriptase negative controls (dH2O and RT -) The housekeeping gene β-Actin is amplified in all samples to ensure RNA integrity and equivalent cDNA template quantity between samples. Relative fold-induction of p15 mRNA following drug treatment, as defined in the *Materials and Methods*, are depicted below their respective lanes. Included in each MSP assay [**(B)** and **(D)**] are unmethylated (HL60) and methylated (METH+) positive controls and water negative controls (dH2O).

MSP. Of note, consistent with previously published data (Cameron et al., 1999a), TSA treatment alone failed to induce p15 expression and promoter demethylation as illustrated previously in Figure 4.11. To our knowledge, this is the first study to combine HDAC inhibitors with zebularine treatment.

4.3.2. Zebularine Mediated Induction of p15 is Associated with Regional Histone Acetylation Enrichment

Previous work on the hypermethylated MLH1 gene in RKO human colon cancer cells has shown that following demethylation by 5-Aza-dC various histone modifications, including H3 acetylation, become enriched at the MLH1 promoter region (Fahrner et al., 2002) and similar results have been reported involving the hypermethylated p16 gene in T24 bladder cancer cells (Nguyen et al., 2002). Based on the literature concerning solid tumor promoter demethylation and regional chromatin remodeling and given our previous data involving 5-Aza-dC induced demethylation of p15 in AML193 cells in association with local enrichment of H3 acetylation, ChIP assays were employed to test if p15 demethylation by zebularine treatment is also associated with local enrichment of acetylated histones.

Using the previously established zebularine dose and treatment time, a ChIP assay was performed on AML193 cells using antibodies for acetylated H3 at lysines 9 and 14, and H4 at lysines 5, 8, 12, and 16 (Fig. 4.14). When the immunoprecipitated DNA was amplified with PCR primers specific for the p15

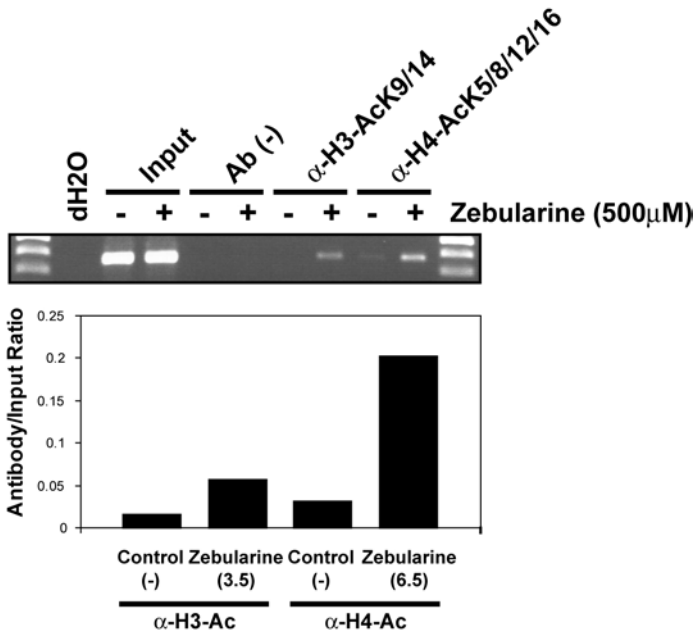

Figure 4.14 ChIP Analysis of H3 and H4 Acetylation Following Zebularine Treatment in AML193 Cells. Illustrated is the ethidium bromide staining of ChIP PCR products from input positive controls (Input), no-antibody negative controls [Ab (-)], acetylated H3 immunoprecipitates (α-H3-AcK9/14), and acetylated H4 immunoprecipitates (α-H4-AcK5/8/12/16). The experiment was performed in the absence and presence of zebularine and included in each PCR is a water negative reagent control (dH2O). The lower panel graphically represents semi-quantitation of the data using densitometry of the PCR products from immunoprecipitated samples normalized to their input counterparts and expressed numerically in brackets as fold change following zebularine treatment.

promoter region (Fig. 4.2) a 3.5 fold enrichment of acetylated H3 and a 6.5 fold enrichment of acetylated H4 was observed following zebularine treatment. These results suggest that like 5-Aza-dC, zebularine also has the ability to affect regional chromatin remodeling, specifically histone acetylation, in association with p15 promoter demethylation.

4.3.3. Zebularine is Cytotoxic and Inhibits AML193 Proliferation

To assess the biological effects of zebularine on cellular toxicity and proliferation, MTT and tritiated thymidine assays were performed. The MTT assay is useful for the quantitation of toxicity and chemosensitivity by measuring mitochondrial metabolism based on the reduction of the MTT tetrazolium salt as a reflection of viable cell number. As previous reports have shown that 5-Aza-dC is most effective at lower doses (Cameron et al., 1999a; Herman et al., 1996b; Issa et al., 2004) and our RTPCR, MSP and ChIP data suggest that zebularine effectively demethylates DNA at dosages in the 100 to 500 μM range (Cheng et al., 2003; Zhou et al., 2002b) we chose 1 and 4 μM 5-Aza-dC and 250 and 500 μM zebularine for MTT analysis. Both 5-Aza-dC and zebularine treatment of AML193 cells greatly reduced the spectrophotometric absorbance in a dose dependent manner when compared to the vehicle control cells (Fig. 4.15A). 5-Aza-dC treatment at 1 and 4 μM resulted in a 69.8% and 76.6% mean reduction in viable cells, respectively, and zebularine treatment at 250 and 500 μM resulted in an 85.2% and 86.6% mean reduction in viable cells, respectively.

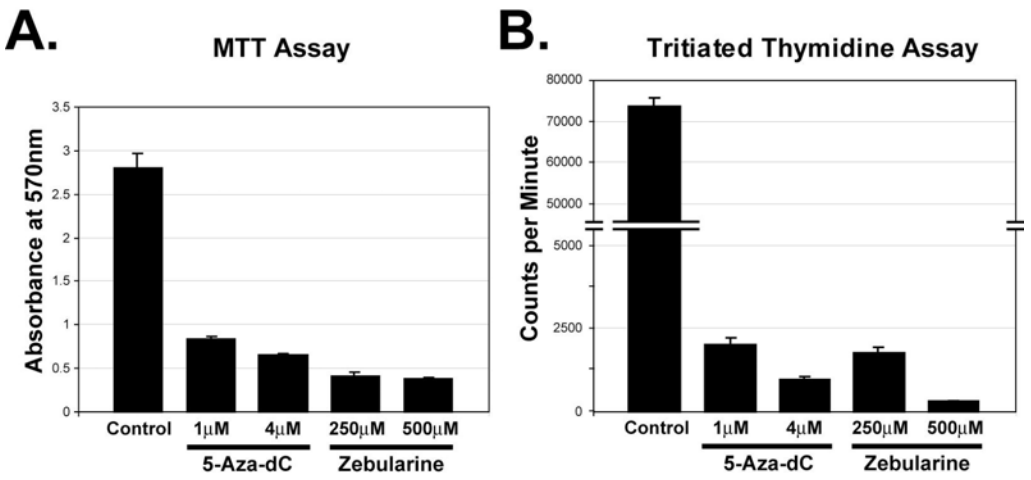

Figure 4.15 MTT and Tritiated Thymidine Assays Following Zebularine Treatment in AML193 Cells. A) MTT assay of AML193 cells following pharmacologic DNMT inhibition. At standard demethylating doses, both 5-Aza-dC and zebularine strongly reduce cell viability. **B)** Tritiated thymidine assay of AML193 cells following pharmacologic DNMT inhibition. At standard demethylating doses, both 5-Aza-dC and zebularine greatly inhibit cellular proliferation.

The tritiated thymidine uptake assay is useful for the quantitation of cellular proliferation by measuring the incorporation of [^{3}H]-labeled thymidine into newly synthesized DNA. Employing the same dosages as used in the MTT assay we found that both 5-Aza-dC and zebularine treatment of AML193 cells resulted in a dramatic reduction in cellular proliferation in a dose dependent manner when compared to the vehicle control cells (Fig. 4.15B). 5-Aza-dC treatment at 1 and 4 μM resulted in a 97.2% and 98.6% mean reduction in cell proliferation, respectively, and zebularine treatment at 250 and 500 μM resulted in a 97.5% and 99.5% mean reduction in cell proliferation, respectively. Taken together, these results show that the cytosine analogs 5-Aza-dC and zebularine, when treated at standard demethylating doses, impart very strong growth inhibitory effects onto AML cells *in vitro* resulting in a decrease in both cell viability and proliferation.

4.3.4. Zebularine Induces Apoptosis and Halts AML193 Cells at G2/M

To test the effects of both 5-Aza-dC and zebularine treatment on the cell cycle, PI DNA staining of AML193 cells was employed. Using the same dosages as the MTT and tritiated thymidine assays we found that both 5-Aza-dC and zebularine treatment of AML193 cells resulted in an increase in the percentage of cells undergoing apoptosis and those arrested in G2/M of the cell cycle (Fig. 4.16). When compared to the vehicle control cells, 1 and 4 μM 5-Aza-dC resulted in a 7.7% and 7.8% increase in apoptotic cells, respectively, while 250 and 500 μM zebularine resulted in a dramatic 16.6% and 25.3% increase in apoptotic cells, respectively. Regarding the G2/M halt, when compared to the vehicle control cells,

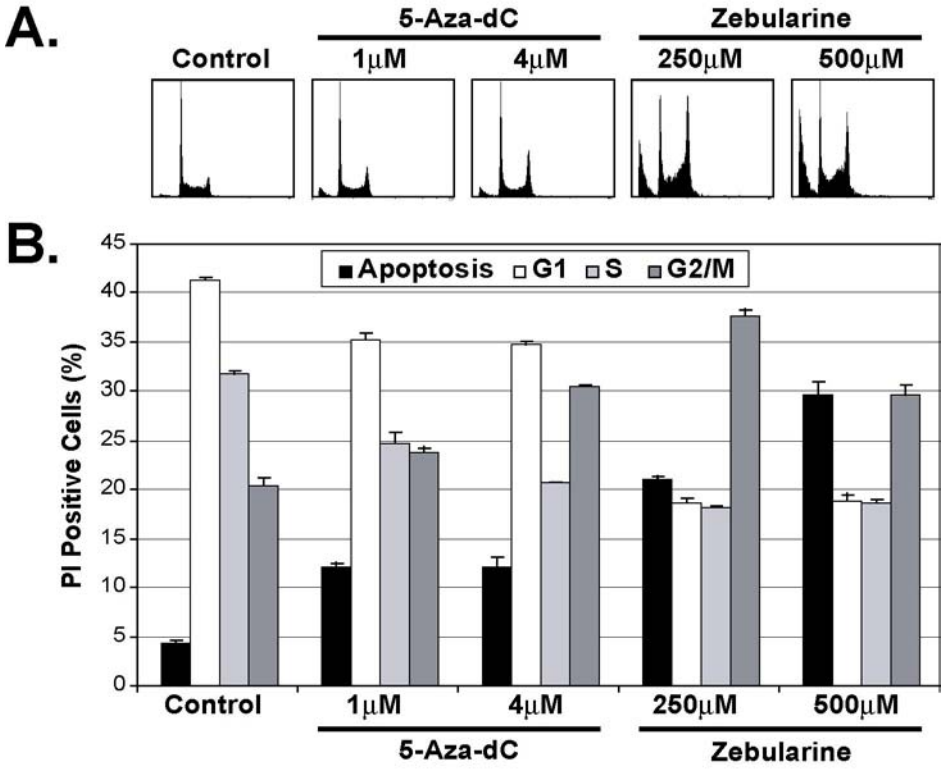

Figure 4.16 Effects of Zebularine on the Cell Cycle in AML193 Cells. A) Histograms of PI stained DNA from AML193 cells before and after pharmacologic DNMT inhibition. **B)** The percentages of cells in the various stages of the cell cycle (see legend) were quantitated from the graphs shown in **(A)** as determined by histogram deconvolution. At standard demethylating doses, both 5-Aza-dC and zebularine increased the number of cells undergoing apoptosis and those halted at G2/M. All results are an average of three independent experiments and error bars represent the standard deviation.

1 and 4 μM 5-Aza-dC resulted in a 3.2% and 10.0% increase in G2/M phase cells, respectively, while 250 and 500 μM zebularine resulted in a 17.1% and 9.1% increase in G2/M phase cells, respectively. The minor increase in G2/M phase cells following 500 μM zebularine (9.1%) when compared to the lower dosage of 250 μM (17.1%) could be a result of the high percentage of cells undergoing apoptosis.

Consistent with the increases in apoptotic and G2/M phase cells, concurrent decreases in the percentage of cells in G0/G1 and S phase were also observed following both 5-Aza-dC and zebularine treatment. The decrease in S phase cells is consistent with the dramatic reduction in cellular proliferation following drug treatment as previously shown by the tritiated thymidine uptake assays. Taken together, these results show that the cytosine analogs 5-Aza-dC and zebularine, when treated at standard demethylating doses, induce apoptosis and G2/M arrest in AML193 cells.

4.3.5. Zebularine Induces Global Histone Phosphorylation in AML193 Cells

As mentioned, the histone code hypothesis has been expanded upon to incorporate such cellular processes as DNA double-strand break repair (Morrison et al., 2004) and apoptosis (Cheung et al., 2003) in addition to the previously hypothesized roles in gene transcription, DNA replication, and chromosome segregation (Jenuwein and Allis, 2001; Strahl and Allis, 2000). Both the extrinsic and the intrinsic pathways of apoptosis result in many molecular events including cleavage of PARP, chromatin condensation, inter-nucleosomal degradation of

DNA, and packaging of the cell into apoptotic bodies. Recently defined histone modifications are also believed to be involved in apoptosis and include the phosphorylation of H2A.X at serine 139 and H2B at serine 14 (Cheung et al., 2003; Rogakou et al., 2000) (Fig. 1.7). H2A.X is rapidly phosphorylated in response to ionizing radiation and other agents that cause DNA double-strand breaks (Rogakou et al., 1998) and is also induced by apoptotic DNA fragmentation (Rogakou et al., 2000). H2B serine 14 phosphorylation has been correlated with cells undergoing programmed cell death (Cheung et al., 2003) and DNA double-strand breakage (Fernandez-Capetillo et al., 2004). Furthermore, although phosphorylation of H3 at serine 10 has traditionally been associated with the induction of mitosis (Prigent and Dimitrov, 2003), evidence does exist for an association with gliotoxin-mediated apoptosis (Waring et al., 1997). As a result of the recent interest in histone phosphorylation as a marker of apoptosis and given the dramatic increase in AML193 cells that undergo apoptosis following zebularine treatment (Fig. 4.16), western blots were employed with antibodies specific for phosphorylated histones following 5-Aza-dC and zebularine time course treatments.

As shown in Figure 4.17, following zebularine treatment, AML193 cells strongly induce global phosphorylation of H3 at serine 10 that is observed as early as two days following initial drug treatment. Phosphorylated serine residues 139 and 14 of H2A.X and H2B, respectively, are also markedly induced in AML193 cells following two days of zebularine treatment that is sustained and enhanced through day six (Fig. 4.17). In comparison to zebularine, minimal induction of H3, H2A.X and H2B phosphorylation is observed following 5-Aza-dC treatment, which

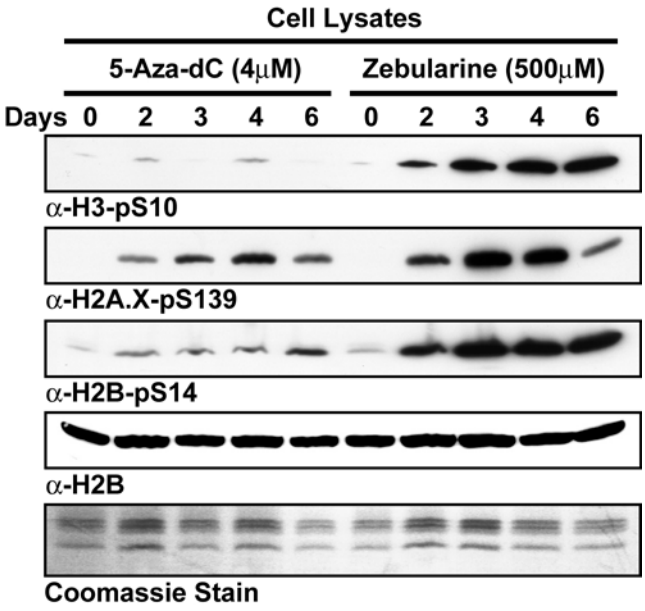

Figure 4.17 Zebularine Induces Global Histone Phosphorylation in AML193 Cells. Time course western blot analysis of phosphorylated and total histone protein levels following treatment with either 5-Aza-dC or zebularine for up to six days. Note the increase in histone and histone variant serine phosphorylation following zebularine treatment when compared to total histone H2B. Parallel gels were stained with Coomassie blue to ensure equivalent sample loading.

correlates with the smaller percentage of cells undergoing apoptosis following 5-Aza-dC treatment illustrated in Figure 4.16. Importantly, the increases in phosphorylated histones are specific and not a reflection of increases in total unmodified histones as confirmed by the equivalent protein bands when the time course assay blot was probed with an antibody for total H2B (Fig. 4.17). Taken together, these results implicate histone phosphorylation of serine residues as a marker of cells undergoing zebularine mediated apoptosis of AML cells *in vitro* and provide further evidence for a potential apoptotic histone code.

4.3.6 DISCUSSION

Hypermethylation of promoter CpG islands, an epigenetic event, represents an alternative mechanism to genomic deletions or mutations in the inactivation of tumor suppressor genes and this frequent alteration in human cancer can potentially lead to the abnormal and uncontrolled growth of cancer cells. Examples of genes that are commonly methylated in cancer include the GSTP1 gene in carcinoma of the prostate (Zhou et al., 2004), the MLH1 gene in colon cancer (Ricciardiello et al., 2003), the RB gene in neuroblastoma (Sakai et al., 1991), the E-cadherin gene in esophageal adenocarcinoma (Corn et al., 2001), and the aforementioned p15 gene in leukemia (Herman et al., 1996b). This type of alteration is of importance to the field of cancer therapeutics as promoter hypermethylation is a reversible process and, consequently, pharmacologic inhibitors of DNA methylation are currently being tested in clinical trials (Egger et al., 2004; Issa, 2003). Several known pharmacological inhibitors exist yet for some their utility as *in vivo* anti-

cancer therapeutics has been limited by toxicity and chemical stability concerns (Beisler, 1978). Previous pre-clinical studies utilizing the novel methylation inhibitor zebularine in solid tumor cell lines and the large collection of data involving p15 promoter hypermethylation in leukemia prompted our investigation of the DNA demethylation and anti-proliferative effects of zebularine in human AML.

Originally developed as an inhibitor of cytidine deaminase (Driscoll et al., 1991; Kim et al., 1986), zebularine contains a 2-(1*H*)-pyrimidinone ring (Fig. 1.9) and has displayed both antitumor and DNA demethylating properties (Cheng et al., 2003; Cheng et al., 2004a; Cheng et al., 2004b; Zhou et al., 2002b). For example, oligonucleotides containing zebularine have been shown to form tight complexes with bacterial methyltransferases that leads to a potent inhibition of DNA methylation (Hurd et al., 1999; Zhou et al., 2002b) and recently Cheng et al. reported a depletion of DNMT1 following zebularine treatment in human T24 bladder cancer cells (Cheng et al., 2004a). In another study by Cheng et al., zebularine was administered orally to EJ6 human bladder cancer-bearing mice and, owing to its stability, was able to significantly reduce tumor growth with minimal toxicity in association with demethylation and re-expression of the p16 gene (Cheng et al., 2003). Our findings of zebularine mediated *in vitro* demethylation and re-expression of the p15 tumor suppressor gene in AML are consistent with the reported data in solid tumor systems and substantiate zebularine as a viable DNA methylation inhibitor in hematological malignancies. Furthermore, this is the first study to our knowledge that has combined zebularine treatment with the HDAC

inhibitor TSA, and like 5-Aza-dC and TSA combinatorial treatment (Cameron et al., 1999a), synergistic induction of p15 mRNA was observed.

As mentioned, the tumor suppressor gene p16, silenced by promoter hypermethylation in T24 bladder cancer cells, has been shown to be reactivated by 5-Aza-dC treatment (Nguyen et al., 2002). Importantly, the authors from this study report that 5-Aza-dC treatment also induced regional chromatin remodeling at this locus, including a release of MeCP2, a reduction in H3 lysine 9 methylation, and enrichment in H3 acetylation and H3 lysine 4 methylation. These results are consistent with our finding that zebularine treatment in AML cells induces p15 mRNA in association with local histone H3 and H4 acetylation, modifications consistent with a chromatin conformation which is accessible to the transcriptional machinery (Agalioti et al., 2002; Morales and Richard-Foy, 2000). Further ChIP assays are necessary to define other proteins and histone modifications that are modulated at this gene locus by zebularine treatment.

Administration of zebularine to AML193 cells caused many anti-proliferative effects including decreased cell viability and proliferation, an increase in the proportion of cells undergoing apoptosis and arrested in G2/M, and a decreased proportion of cells in G0/G1 and S phases of the cell cycle. These results were comparable to 5-Aza-dC yet were often more dramatic, possibly as a result of the higher dosages required to efficiently demethylate DNA and reconstitute p15 expression. A recent study using bile duct cancer cells also found 5-Aza-dC to be cytotoxic with an increase in apoptotic cells, yet found the cells to be halted in the G0/G1 phase of the cell cycle (Tang et al., 2004). Similarly, Yang

et al. reported 5-Aza-dC mediated growth inhibition in breast cancer cells with an increase in G0/G1, not G2/M, phase cells (Yang et al., 2002). However, a very early study of 5-Aza-dC in HL60 human leukemic cells found, similar to the data presented herein, that 5-Aza-dC administration resulted in an increased proportion of cells in G2/M rather than G0/G1 (Levva et al., 1986). This implies that cell cycle responses to DNA methylation inhibition may be cell type specific. The difference in cellular response to DNMT inhibition is understandable as different genes will be induced in different cell types following 5-Aza-dC and/or zebularine treatment depending on which genes are silenced by promoter hypermethylation. Global gene expression arrays would be useful to test this hypothesis.

To this end a microarray study using zebularine in the KAS-6/1 multiple myeloma cell line has recently revealed a number of genes to be demethylated and re-expressed following drug treatment which have the potential to significantly contribute to myeloma cell growth and survival (Pompeia et al., 2004). Furthermore, the preference of zebularine incorporation into cancer cells, as opposed to normal fibroblasts, has recently been reported in a microarray study by Cheng et al. which showed considerably more affected genes in bladder, colon, and pancreatic carcinoma cells then the normal fibroblasts (Cheng et al., 2004b). This study also reported a decrease in detectable DNMT1 protein following zebularine administration similar to the aforementioned study that used bladder cancer cells (Cheng et al., 2004a) and has also been reported following 5-Aza-dC treatment (Velicescu et al., 2002). This is presumably a result of the 'quenching' effect that

the cytosine analogs have on DNMTs when incorporated into newly synthesized DNA (Bouchard and Momparler, 1983; Santi et al., 1983; Santi et al., 1984).

Outside of the DNMTs, no other global protein levels have been analyzed to date following *in vitro* zebularine administration. The growing interest in the histone code, its relation to DNA hypermethylation (Fahrner et al., 2002), and the hypothesis that an apoptotic histone code exists (Cheung et al., 2003) prompted our analysis of global protein levels of phosphorylated histones, specifically H3-phosphoserine 10, H2A.X-phosphoserine 139, and H2B-phosphoserine 14, following zebularine treatment. Consistent with the increases observed in the percentage of cells undergoing apoptosis following zebularine treatment, global induction of histone phosphorylation was detected in AML cells within two days of drug administration. The role of histone phosphorylation during apoptosis is still unresolved yet it is attractive to hypothesize that histone modifications, such as serine phosphorylation, play functional roles in promoting the drastic changes that occur in DNA integrity and chromatin compaction during apoptosis. Whether these modifications have functional consequences regarding apoptotic chromatin condensation or are merely DNA damage markers remains to be determined.

Taken together, our findings indicate that zebularine is an effective inhibitor of p15 promoter hypermethylation and cell growth in human AML *in vitro*. By expanding the spectrum of zebularine antitumor effects to include hematological malignancies, our study also provides further support for the clinical potential of this agent as a cancer therapy, alone, or in combination with other drugs such as 5-Aza-dC and HDAC inhibitors.

4.4. cDNA MICROARRAY ANALYSIS OF GENES UPREGULATED BY TREATMENT WITH 5-Aza-dC IN COMBINATION WITH TSA IDENTIFIES ABERRANT MT1H PROMOTER HYPERMETHYLATION AT A HIGH FREQUENCY IN HUMAN AML

4.4.1. cDNA Microarray Analysis of AML193 Cells Following DNMT and HDAC Inhibition

As mentioned, it is well documented that a common occurrence in cancer cells is the aberrant hypermethylation of CpG islands in gene promoters with accompanying loss of transcription (Baylin and Herman, 2000). Although many genes have been identified which are frequently affected by promoter hypermethylation in specific types of cancer, numerous experimental strategies have been utilized in the hope of discovering novel genes affected by aberrant epigenetic silencing. One strategy that has been employed for this purpose combines high throughput gene expression analysis with pharmalogical inhibition of the epigenetic silencing machinery. Based on the recent success of this approach in colorectal cancer (Suzuki et al., 2002), esophageal squamous cell carcinoma (Yamashita et al., 2002), pancreatic carcinoma (Sato et al., 2003a), and multiple myeloma (Pompeia et al., 2004), and given the fact that multiple genes have been shown to be concurrently hypermethylated in human cancer including AML (Esteller et al., 2001; Melki et al., 1999; Paz et al., 2003), cDNA microarray analysis was performed on AML193 cells following treatment with 5-Aza-dC and

TSA in order to identify novel genes aberrantly silenced by promoter hypermethylation in AML.

As the previous work with this drug combination successfully demethylated and synergistically upregulated the CDKI p15 in AML193 cells, the same drug dosages and incubation times were employed to screen for novel genes silenced by promoter hypermethylation. Following microarray analysis, genes with a $\log_2$ ratio greater than 1 in all three microarrays following treatment with 5-Aza-dC and TSA were considered upregulated and are listed in Table 4.1. Seven genes were found to be consistently upregulated by cDNA microarray analysis and all have known chromosomal locations and transcriptional start sites as defined by public genome databases. Of the 1718 genes spotted on the 1.7K human microarrays, 978 were used in the data analysis based on their conforming to the filtering criteria between each of the three microarrays. Criteria to incorporate specific microarray genes into the analysis included, but was not limited to, spot morphology, minimum intensity values, and consistency between each of the three microarray experiments. The seven genes that were successfully identified by the microarray screen were analyzed by semi-quantitative RTPCR in cells subjected to vehicle treatment, 5-Aza-dC alone, TSA alone, or a combination of the two drugs (Fig. 4.17).

The upregulation of metallothionein 1H (MT1H), metallothionein 1G (MT1G), spermidine/spermine N1-acetyltransferase (SAT), metallothionein 1E (MT1E), interferon-gamma inducible protein 30 (IFI30), glutathione peroxidase 4 (GPX4), and metallothionein 2A (MT2A), as detected by microarray, following combinatorial drug treatment was confirmed by RTPCR in all cases. Interestingly,

Table 4.1 Genes Upregulated by 5-Aza-dC and TSA Treatment in AML193 Cells

Accession No.[a]	Gene Name	Symbol	Location	CpG Island[b]	Meth.[c]
R93527	Metallothionein 1H	**MT1H**	16q13	yes	yes
NM_005950	Metallothionein 1G	**MT1G**	16q13	yes	yes
AA056280	Spermidine/Spermine N1-Acetyltransferase	**SAT**	Xp22.1	yes	partial
H93255	Metallothionein 1E	**MT1E**	16q13	yes	partial
W24688	Interferon-Gamma Inducible Protein 30	**IFI30**	19p13.1	yes	no
N31188	Glutathione Peroxidase 4	**GPX4**	19p13.3	yes	no
H91613	Metallothionein 2A	**MT2A**	16q13	yes	no

[a] GenBank accession number.
[b] yes: CpG island located near presumed transcription start site or 5' upstream region; no: no CpG island located near presumed transcription start site or 5' upstream region.
[c] yes: fully methylated; partial: partially methylated; no: no methylation detected.

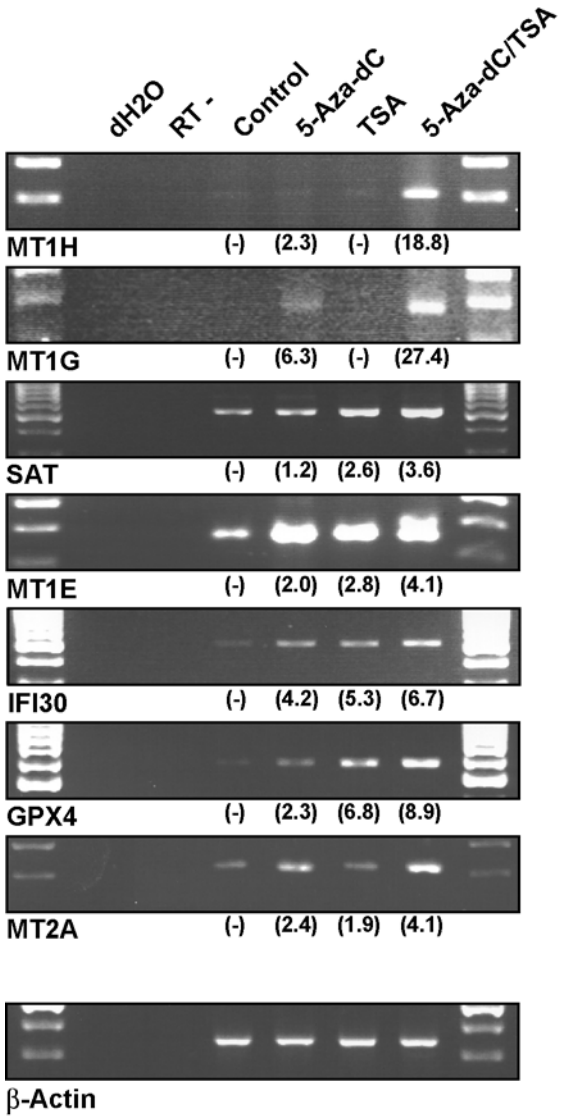

Figure 4.18 Semi-quantitative RTPCR of Candidate Genes Identified by cDNA Microarrays. The seven genes identified by microarray analysis and the housekeeping gene β-Actin were subjected to RTPCR analysis following control, individual, and combinatorial drug treatments. The fold change in target gene mRNA, as compared to the vehicle control, is represented numerically in brackets below individual sample gel lanes.

the degree of transcriptional upregulation following individual drug treatment varied and in some cases (SAT, MT1E, IFI30, GPX4, MT2A) basal mRNA was detected in the vehicle treated cells. However, in almost all cases, treatment with 5-Aza-dC alone resulted in an induction of detectable candidate gene mRNA, implying the possibility of promoter hypermethylation mediated silencing at these loci. Moreover, the gene MT1G displayed a classic synergistic upregulation pattern following drug treatments, similar to the previously studied CDKI p15, further reinforcing the rationale for downstream methylation analysis.

4.4.2. Methylation Analysis of Candidate Genes in AML193 Cells

All seven genes upregulated by treatment with 5-Aza-dC and TSA harbored CpG islands, as defined by Takai and Jones (Takai and Jones, 2002), within their 5' promoter regions. Given the availability of the 5' promoter sequence of all seven candidate genes, MSP primers were designed to test for the presence of CpG island hypermethylation. Limiting the analysis to AML193 cells, out of the seven candidate genes, four harbored methylated promoter alleles (MT1H, MT1G, SAT, MT1E) and the remaining three genes (IFI30, GPX4, MT2A) amplified strictly unmethylated alleles (Fig. 4.18). Interestingly, SAT and MT1E displayed a partial methylation pattern as they harbored both unmethylated and methylated alleles (Fig. 4.18B). Importantly, the two genes with complete promoter hypermethylation (MT1H, MT1G) were partially demethylated following a 72 hour treatment with 5-Aza-dC (4.18A). This timeframe is consistent with 5-Aza-dC induced demethylation of p15 and provides further evidence of promoter hypermethylation

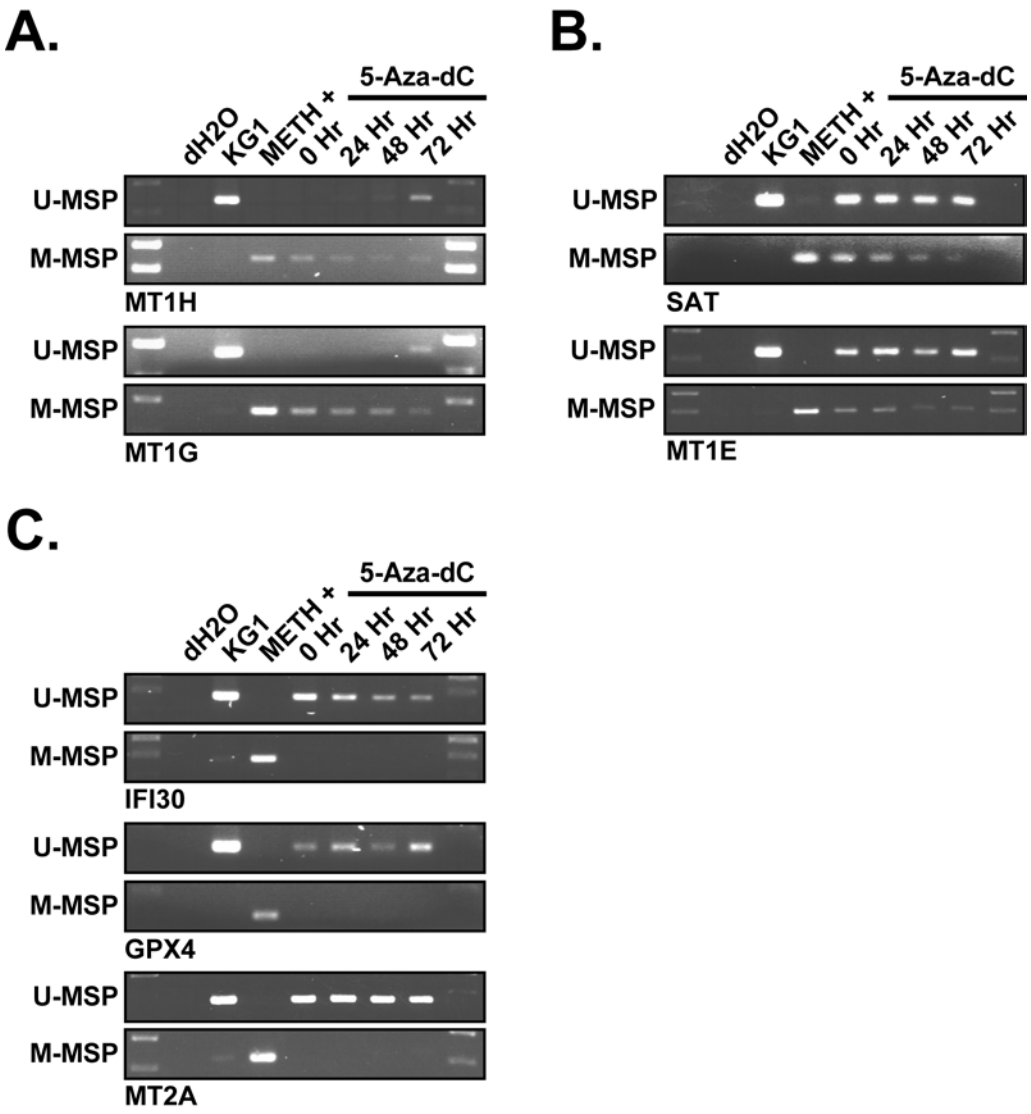

Figure 4.19 MSP of Candidate Genes Following Treatment with 5-Aza-dC. **A)** MSP analysis of Group 1 genes (MT1H, MT1G) harboring completely methylated alleles. Note the reconstitution of unmethylated alleles following 72 hours of 5-Aza-dC treatment. **B)** MSP analysis of Group 2 genes (SAT, MT1E) harboring partially methylated alleles. **C)** MSP analysis of Group 3 genes (IFI30, GPX4, MT2A) harboring completely unmethylated alleles. Commercially available universally methylated bisulfite modified DNA was used as a methylated positive control (METH +) and water as a negative reagent control (dH2O) for all reactions.

mediated gene silencing at these loci. Taken together, the seven candidate genes were categorized into three groups based on their promoter methylation patterns in AML193 cells: Group 1 genes harbored completely methylated promoter alleles (MT1H, MT1G); Group 2 genes harbored partially methylated promoter alleles (SAT, MT1E); and Group 3 genes harbored completely unmethylated promoter alleles (IFI30, GPX4, MT2A) (Fig. 4.18 and Table 4.1).

4.4.3. Methylation Analysis of Metallothionein Genes in AML

The most striking finding from the microarray analysis was that of the seven candidate genes, four (MT1H, MT1G, MT1E, MT2A) are members of the metallothionein family of cysteine-rich small molecular weight proteins. The majority of this gene family is located on chromosome 16q13 (West et al., 1990) (Fig. 4.20), and although their function has yet to be fully realized, they are induced by toxic heavy metals, UV irradiation, and reactive oxygen species and are considered to be important mediators of cellular detoxification (Palmiter, 1998). Many of the metallothioneins at this locus have promoter region CpG islands and, interestingly, MT1G promoter hypermethylation has recently been implicated in the pathogenesis of sporadic papillary thyroid carcinoma (Huang et al., 2003). As such, the metallothionein genes that were identified by microarray analysis of AML193 cells (MT1H, MT1G, MT1E, MT2A) were subsequently analyzed for promoter hypermethylation in a panel of AML cell lines.

Including AML193 cells, promoter hypermethylation was detected in MT1H, MT1G, MT1E and MT2A in 5/7 (71%), 2/7 (29%), 2/7 (29%), and 0/7

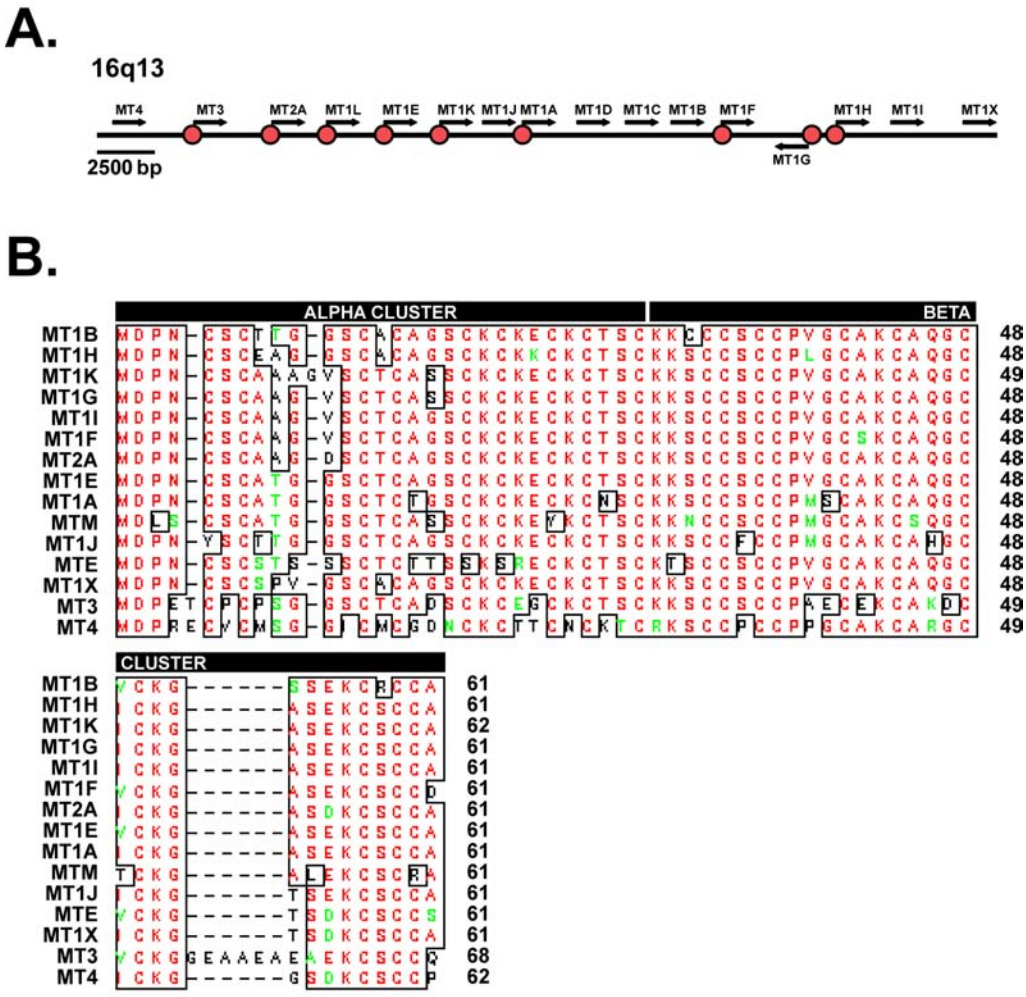

Figure 4.20 16q13 Locus and Metallothionein Amino Acid Alignment. A) Schematic of metallothionein genes located at 16q13. Arrows represent gene location and orientation and red circles represent known CpG islands. **B)** The amino acid sequences of known metallothioneins are aligned revealing both the high degree of homology and the cysteine-rich alpha and beta clusters (represented by black bars above sequence). Alignments were generated using the 'ClustalW' amino acid sequence alignment algorithm and the 'prettyplot' diagram tool available from EMBOSS.

(0%) AML cell lines, respectively (Fig. 4.21A). Interestingly, promoter hypermethylation was detected in three of the candidate genes (MT1H, MT1G, MT1E) in KG1A and AML193 cells, which also both harbor complete p15 promoter hypermethylation (Fig. 4.3), implying the possibility of a common abnormality in the DNA methylation machinery of these cell lines. To investigate for the occurrence of MT1H, MT1G and MT1E promoter hypermethylation in human AML *in vivo*, AML patient blasts (n = 39) and non-leukemic controls (n = 13) were analyzed by MSP. MT1H, MT1G and MT1E methylation was detected in 20/39 (51%), 1/37 (3%) and 0/37 (0%) AML patient samples (Fig. 4.20B), respectively, and there was no methylation of any of these genes in control samples (Fig. 4.20C). Taken together, MT1H, MT1G and MT1E promoter hypermethylation was identified in 27/46 (54%), 3/44 (7%) and 2/44 (5%) AML cell lines and patient samples, respectively.

4.4.4. 5-Aza-dC Mediated Induction of MT1H is Associated with Changes in H3 Methylation and Acetylation

To further address our hypothesis, we chose to investigate the MT1H promoter for changes in histone modifications following 5-Aza-dC mediated promoter demethylation. Given our previous analysis of the methylated p15 promoter in AML193 cells which identified deacetylated histones in association with specific H3 lysine methylation, ChIP assays were employed to determine if similar patterns of H3 amino-terminal modifications were present at the hypermethylated MT1H promoter region in AML193 cells. As illustrated in Figure

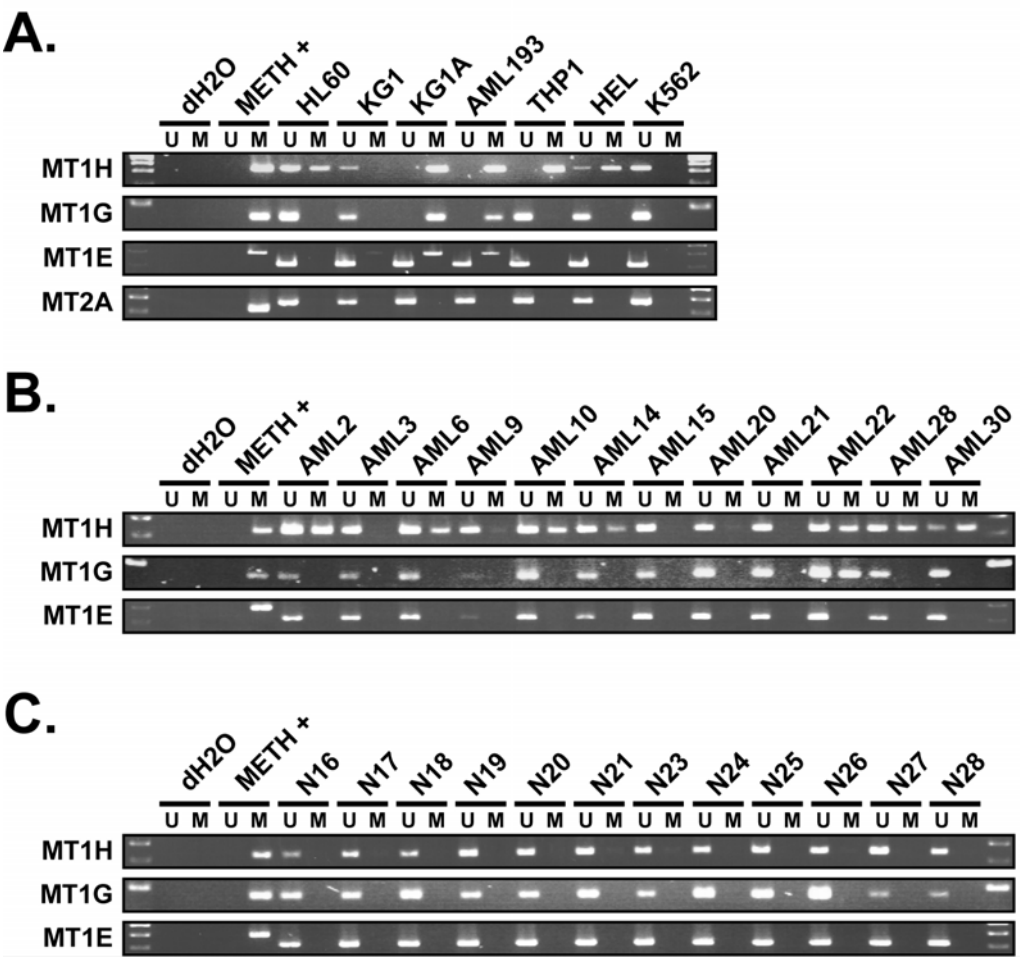

Figure 4.21 Metallothionein Promoter Hypermethylation. **A)** MSP analysis of bisulfite modified DNA from AML cell lines. **B)** Representative MSP analysis of bisulfite modified DNA from AML patient samples. **C)** Representative MSP analysis of bisulfite modified DNA from non-leukemic control samples. Commercially available universally methylated bisulfite modified DNA was used as a methylated positive control (METH +) and water as a negative reagent control (dH2O) for all reactions. U: unmethylated MSP reaction; M: methylated MSP reaction.

4.22, the MT1H promoter is associated with methylated H3 lysine 9 and deacetylated H3 at lysines 9 and 14 in AML193 cells and, like p15, following 5-Aza-dC mediated demethylation, both modifications are reversed. This finding is consistent with our model of promoter hypermethylation mediated p15 gene silencing and further implicates specific histone modification patterns in the remodeling of chromatin to affect epigenetic gene silencing. Importantly, similar H3 modifications have been observed at the hypermethylated metallothionein I promoter in rat lymphosarcoma cells which were also reversible following treatment with 5-Aza (Ghoshal et al., 2002). Further ChIP assays are warranted to investigate which other histone modifications are associated with the methylated MT1H promoter in AML.

4.4.5. DISCUSSION

Since the discovery that DNMT and HDAC inhibitors synergistically induce transcription of genes silenced by promoter hypermethylation *in vitro* (Cameron et al., 1999a), numerous groups have exploited this model to further understand the mechanism behind epigenetic silencing. As a result, it is now commonplace to reactivate epigenetically silenced tumor suppressor genes *in vitro* with pharmacological inhibitors of both the DNMT and HDAC machineries and this combination of drugs is currently being tested in a clinical setting (Shaker et al., 2003). Recently, DNMT inhibitors such as 5-Aza and 5-Aza-dC have been utilized in the clinic for the treatment of hematological malignancies (Lyons et al., 2003) and solid tumors (Aparicio et al., 2003) with varying outcomes. A phase I

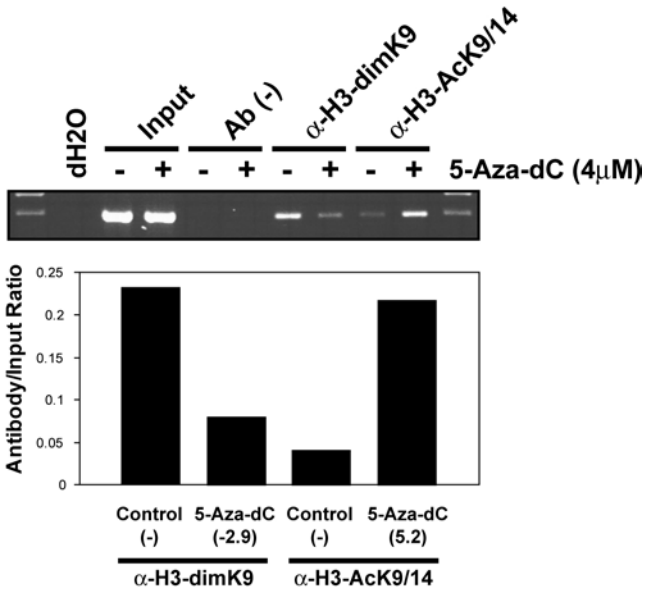

Figure 4.22 MT1H ChIP Analysis of H3 Lysine 9 Methylation and Acetylation. Illustrated is the ethidium bromide staining of ChIP PCR products from input positive controls (Input), no-antibody negative controls [Ab (-)], methylated H3 immunoprecipitates (α-H3-dimK9), and acetylated H3 immunoprecipitates (α-H3-AcK9/14). The experiment was performed in the absence and presence of 5-Aza-dC and included in each PCR is a water negative reagent control (dH2O). The lower panel graphically represents semi-quantitation of the data using densitometry of the PCR products from immunoprecipitated samples normalized to their input counterparts and expressed numerically in brackets as fold change following 5-Aza-dC treatment.

study involving solid tumors reported no objective responses in patients with metastatic disease yet 5-Aza-dC was well tolerated and changes in gene methylation were observed (Aparicio et al., 2003). In a recent phase I trial analyzing the efficacy of 5-Aza-dC in the treatment of multiple hematological cancers, the drug was well tolerated and appeared to be more effective at low, not high, doses (Issa et al., 2004). Based on the recent resurgence of interest in DNMT inhibitors in the treatment of cancer and given that in diseases such as AML, promoter hypermethylation is most often not limited to a single gene, but rather a number of genes concurrently (Melki et al., 1999), a microarray study was designed to identify novel genes aberrantly silenced by promoter hypermethylation in human AML.

Other research groups have identified novel genes silenced by promoter hypermethylation in diverse cancer types using a number of different experimental strategies. The present study employed cDNA microarray expression analysis following pharmacological inhibition of both the DNMT and HDAC machineries in the human cell line AML193 and out of 978 genes analyzed, seven (MT1H, MT1G, SAT, MT1E, IFI30, GPX4, MT2A) were found to be consistently upregulated. The percentage of genes upregulated in the present study (0.7%) is comparable to the previous studies that utilized a similar methodology. Following combinatorial drug treatment, the colorectal cancer study (Suzuki et al., 2002), esophageal squamous cell carcinoma study (Yamashita et al., 2002), and the pancreatic carcinoma study (Sato et al., 2003a) identified upregulation in 0.7%, ~0.5%, and ~1.6%, of the total genes analyzed, respectively.

All genes identified by the microarray screen in the present study were confirmed by RTPCR and subsequent MSP analysis revealed promoter hypermethylation in four of the seven genes identified (MT1H, MT1G, SAT, MT1E). Importantly, following treatment with 5-Aza-dC the two genes that harbored fully methylated alleles (MT1H, MT1G) reconstituted unmethylated alleles in association with the induction of detectable mRNA. The three genes that did not harbor promoter hypermethylation (IFI30, GPX4, MT2A) may have been upregulated at the mRNA level as a secondary effect from an upstream epigenetically regulated gene. Or, in the case of MT2A, the high degree of nucleotide homology between it and its metallothionein family members may be responsible for triggering a false positive result. Moreover, a number of unmethylated genes have been previously reported to be induced by DNMT inhibition in other microarray studies and more detailed investigation regarding the methylation independent activities of 5-Aza-dC has been put forward regarding the unmethylated CDKIs p19 (Zhu et al., 2001) and p21 (Lavelle et al., 2003).

Strikingly, of the seven candidate genes identified by microarray, four are members of the metallothionein family of cysteine-rich small molecules located at 16q13 and three of the four (MT1H, MT1G, MT1E) were shown to harbor promoter hypermethylation in the AML193 cell line. Many isoforms of human metallothioneins exist, including non-functional pseudogenes (Stennard et al., 1994; West et al., 1990), rendering them difficult to study due to their high degree of conservation at both the amino acid and nucleotide level. Although the function of metallothioneins likely involves both metal ion homeostasis and metal ion

chaperoning for the synthesis of other metalloproteins, their role in cancer is largely unknown (Theocharis et al., 2004).

Based on the ability of metallothioneins to neutralize free radicals, it has been suggested that these proteins may play a role in the suppression of the toxic effects of mutagens and anticancer agents by protecting the DNA from alkylation (Waalkes et al., 1996). This is further evidenced by the fact that transcriptional upregulation of metallothioneins following irinotecan treatment, a chemotherapeutic DNA topoisomerase I inhibitor, is associated with drug resistance in a small set of gastric cancer patients (Chun et al., 2004). Furthermore, there is direct evidence that certain tumors have enhanced levels of metallothionein proteins and that induction of these metalloproteins is often associated with rapidly proliferating cells (Cherian et al., 1993). Examples of tumors that have shown elevated metallothionein expression by immunohistochemistry include thyroid carcinoma (Nartey et al., 1987), testicular germ cell tumors (Chin et al., 1993), and bladder transitional cell carcinoma (Bahnson et al., 1991). Given that the majority of metallothionein expression data originates from early studies on solid tumors, the exact role metallothioneins may play in hematological malignancies is as yet unknown.

Although previous studies emphasize upregulation and overexpression of metallothioneins in association with malignancy, compelling evidence from recent reports implicates metallothionein transcriptional *inactivation* and associated promoter hypermethylation in mouse lymphosarcoma (Ghoshal et al., 2002), rat hepatoma (Ghoshal et al., 2000; Majumder et al., 2002), human gastric cancer

(Deng et al., 2003), and papillary thyroid carcinoma cells (Huang et al., 2003). These reports, and the data presented herein, suggest that the absence of metallothionein protein(s) may be associated with tumorigenesis. For example, Deng et al. report metallothionein 3 (MT3) 5' CpG island hypermethylation in gastric cancer cell lines in association with undetectable mRNA that is reversed following treatment with 5-Aza (Deng et al., 2003). Furthermore, the authors found a significantly higher degree of MT3 hypermethylation in primary tumor samples when compared to non-malignant gastric epithelial tissues. Huang et al. recently reported MT1G promoter hypermethylation in association with low mRNA expression in papillary thyroid cell lines and patient samples (Huang et al., 2003) and using dosages similar to the present study, the authors report marked MT1G induction following treatment with 5-Aza-dC and TSA. Of note, MT1G promoter hypermethylation was also identified in 29% of our AML cell lines and, interestingly, was consistently detected in conjunction with neighboring MT1H promoter hypermethylation. As the two genes are orientated in opposite directions with CpG islands separated by a mere kilobase (Fig. 4.19A), it is attractive to hypothesize that a methylation 'hotspot' exists at this locus.

Further evidence of methylation mediated metallothionein inactivation in cancer cells comes from two previous studies involving cDNA microarray analysis following pharmacological DNMT inhibition. The esophageal squamous cell carcinoma study by Yamashita et al. also identified MT1G promoter hypermethylation (Yamashita et al., 2002) and a recent microarray study by Schuster et al. identified MT1B and MT1L mRNA upregulation following 5-Aza-

dC treatment in both breast and liver carcinoma cell lines (Schuster et al., 2003). Importantly, in the latter study, the authors also reported 5-Aza-dC induced mRNA upregulation of SAT in a glioblastoma cell line and IFI30 upregulation in prostate and breast carcinoma cell lines. Although these genes were also found to be upregulated in our microarray dataset, it should be noted that no confirmation of DNA methylation was performed in the solid tumor cell line study. As such, the induction of IFI30, as evidenced in our study, may not be a direct result of promoter demethylation but rather a secondary effect.

The finding of SAT partial methylation in our data is not completely surprising as the gene is located near the pseudoautosomal region of the X chromosome and, like most X-linked genes in females, is imprinted, only actively transcribing one allele (Mank-Seymour et al., 1998). Partial methylation of the SAT gene in our data could be indicative of monoallelic methylation, a hallmark of X chromosome inactivation. Furthermore, the cells used in the aforementioned study (U-87 glioblastoma cells) and those used in the present study (AML193 cells) are both derived from females and therefore could be normally regulated by DNA methylation mediated imprinting. Further SAT methylation studies with germline female DNA controls are required to confirm these observations.

Taken together, the current base of scientific literature reinforces our findings of MT1H promoter hypermethylation in AML and suggests that repression of metallothionein transcription may play a role in promoting tumor cell growth in specific cell types. Further studies are required to elucidate the exact role these proteins play in cellular transformation and to what effect metallothionein

repression has on heavy metal cellular homeostasis. Our findings implicate MT1H promoter hypermethylation in the pathogenesis of human AML and suggest that the use of cDNA microarray technology following pharmacological manipulation is a useful approach for identifying novel epigenetically silenced genes in this disease.

5. CONCLUSIONS AND FUTURE STUDIES

The etiology of human AML for any individual patient is unknown. In some cases it is a disease secondary to an underlying bone marrow disorder, either congenital or acquired, and in the remaining patients the disease arises spontaneously. To address this, a substantial effort has been put forward to assemble a better understanding of AML pathogenesis and the increasing body of knowledge regarding the genetic components of AML is beginning to direct a promising array of targeted therapies, many of which are in clinical development. Yet, despite such optimism, the majority of AML patients will die of their disease. As such, a major component of the research compiled in this thesis revolves around the need to better understand the epigenetic components involved in the pathogenesis of AML. The specific aims of this project were defined with the goal of studying epigenetically silenced genes potentially involved in AML pathogenesis and the cytosine analogs currently used to inhibit or reverse this process.

A major finding of the work presented in this thesis is the high frequency of p15 promoter hypermethylation in association with absent p15 mRNA in AML patients and cell lines. Importantly, the CDKI methylation data in this study was evaluated using not only conventional MSP, but also a novel technique known as

TTGE. This technique has previously been used for mutation detection yet technical modifications designed in our laboratory have allowed for the detailed analysis of DNA methylation (Scott et al., 2003; Scott et al., 2004). The technique enables visualization of promoter hypermethylation heterogeneity and was adapted from a similar study which used DGGE (Aggerholm et al., 1999). To the best of our knowledge, our lab is the first to utilize TTGE for the analysis of DNA methylation.

The CDKI genes analyzed in this study are all downstream mediators of the TGF-β1 pathway. The TGF-β1 cytokine is important as it can trigger a diverse set of responses, including inhibition of cell cycle progression, facilitation of differentiation, induction of apoptosis, and regulation of cell adhesion and extracellular matrix production, depending on the genetic makeup and environment of the target cell (Blobe et al., 2000; Massague, 2000). A natural extension from the studies presented in this thesis would therefore be to investigate other non-TGF-β1 pathway members of both the CIP/KIP and INK4 families of CDKIs for promoter hypermethylation and transcriptional silencing. For example, the CIP/KIP candidate tumor suppressor gene p57 has recently been implicated in promoter hypermethylation mediated gene silencing in a number of tumor types (Kikuchi et al., 2002; Li et al., 2002b; Shen et al., 2003) and could therefore be quickly analyzed in our panel of AML samples. Furthermore, the other INK4 genes p16, p18, and p19 have all been associated with epigenetic silencing in other cancers and could also be candidates for aberrant silencing in AML. Of note, preliminary data on the p16 promoter associated CpG island in our sample set has

revealed p16 methylation mediated silencing in only a small percentage of AML patients and cell lines (data not shown).

Importantly, no promoter hypermethylation was detected in the CpG island of the CDKI p21 in any of the AML samples used in this study, even those that lacked detectable p21 mRNA. Given the paucity of mutations reported in this gene and the growing body of literature implicating histone deacetylation as the primary cause of aberrant p21 gene silencing in cancer (Gui et al., 2004; Richon et al., 2000), further work is warranted to characterize the mechanism behind p21 methylation independent silencing in human AML and its potential as a target for epigenetic therapy. To this end, work in our lab is ongoing. Recent data supports the hypothesis that the demethylating agent 5-Aza-dC has methylation independent activity as we have found that 5-Aza-dC relieves p21 repression in AML cell lines by a mechanism involving release of HDAC1 and enrichment of local H3 acetylation (manuscript submitted for publication). We also found that combined 5-Aza-dC and HDAC inhibitor treatment enhances chromatin remodeling and reactivation of the unmethylated p21 tumor suppressor gene. This latter finding is of relevance to the clinical use of these agents in AML due to the fact that the p21 promoter is unmethylated *in vivo*.

The burgeoning relationship between promoter hypermethylation and histone amino-terminal modifications is also worth pursuing. Our finding of a repressor complex that includes MeCP2, SUV39h1, deacetylated H3, methylated H3 lysine 9, and depleted H3 lysine 4 and arginine 17 methylation in association with p15 promoter hypermethylation is novel and it would be interesting to identify

which other proteins are involved at this region (HDACs, HP1, other chromatin remodeling factors, etc.) and which other histone modifications are consistent with transcriptional inactivity, including those on H4. Further ChIP assays, given the availability of high quality antibodies, are required to address these types of questions. Moreover, unknown transcription factors and epigenetic repressors could also be identified by combining ChIP assays with modern mass spectrometry techniques. Although this latter methodology would not be able to identify proteins at specific genomic loci, such as promoter regulatory regions, it could assist in the identification of unknown protein complexes involved in epigenetic transcriptional repression.

It is important to emphasize the ability of 5-Aza-dC in our data to not only demethylate promoter regions and relieve transcriptional repression, but also its ability to facilitate the release of MBD proteins and reverse the H3 modifications normally associated with gene silencing. 5-Aza-dC is becoming increasingly attractive as a therapeutic option for patients with hematological malignancies (Daskalakis et al., 2002; Issa et al., 2004; Kantarjian et al., 2003) and it is imperative to have a full understanding as to the mechanisms behind its tumor suppressive and gene inducing activities. Future work is required to identify the target genes of this agent, both methylation dependent and independent, and to increase the tumor cell specificity of this global acting drug. Moreover, the identification of other less toxic DNMT inhibiting agents and their evaluation both *in vitro* and *in vivo* will be of great value.

To address this issue, a significant portion of the work reported in this thesis was devoted to the study of a relatively new DNMT inhibitor, zebularine, using AML193 cells as a model system. Zebularine was able to demethylate the p15 promoter and induce transcription with local enrichment of histone acetylation, but only at significantly higher doses than those required for 5-Aza-dC mediated p15 induction. However, owing to the *in vivo* stability and activity of zebularine (Cheng et al., 2003) and given the conflicting reports on the ability of 5-Aza-dC to demethylate p15 *in vivo* (Daskalakis et al., 2002; Issa et al., 2004), it will be interesting to follow the pre-clinical evaluation of zebularine as it becomes a more utilized DNMT inhibitor in hematological malignancies, both *in vitro* and *in vivo*.

The ability of pharmacological agents to reactivate epigenetically silenced genes was exploited in this study in order to identify novel genes silenced by aberrant promoter hypermethylation in AML. Using cDNA microarrays seven genes were found to be consistently upregulated following treatment with 5-Aza-dC and TSA in AML193 cells. Of these candidate genes, detailed MSP analysis identified a high frequency of promoter hypermethylation in a number of metallothionein genes in AML cell lines and further experimentation identified one gene, MT1H, to be hypermethylated at a high frequency in AML patient samples. Remarkably, metallothionein induction following 5-Aza-dC treatment was recognized in rat liver cells almost twenty years ago prior to the identification of promoter methylation mediated metallothionein transcriptional silencing (Waalkes et al., 1988).

However, although the microarray approach was very successful in identifying genes silenced by promoter hypermethylation, the exact role that these genes play in AML pathogenesis, if any, is unknown. Given that similar approaches in other cell types have also identified aberrant metallothionein methylation (Schuster et al., 2003; Yamashita et al., 2002), it would be of value to extend these studies further to determine if metallothioneins are bona fide tumor suppressor genes in AML. Studies in our lab are currently underway to test the effects of promoter hypermethylation on MT1H induction following treatment with cadmium, a heavy metal known to rapidly induce metallothionein expression (Ghoshal et al., 1998; Majumder et al., 1999). As such, we hypothesize that promoter hypermethylation will inhibit cadmium mediated induction of the MT1H gene in AML cells and that 5-Aza-dC pretreatment, and therefore MT1H demethylation, will re-enable cadmium induced MT1H expression.

Another approach to identifying novel genes silenced by promoter hypermethylation combines ChIP assays with commercially available CpG island microarrays. This technique has been previously used to identify specific transcription factor targets (Weinmann et al., 2002) and genes associated with specific histone modifications, notably H3 lysine 9 acetylation and methylation (Kondo et al., 2004). By immunoprecipitating with high quality antibodies for mammalian DNMTs, either DNMT1 and/or DNMT3b, one could identify CpG islands associated with the DNA methylation machinery, therefore most likely identifying hypermethylated promoter loci. Confirmation of candidate genes could be accomplished by standard bisulfite-based techniques and gene expression

correlation by RTPCR. It would be interesting to perform this type of experiment in AML193 cells as it could potentially provide valuable confirmation to the presented cDNA microarray data.

In conclusion, the findings presented within this thesis provide new insights into aberrant epigenetics in the molecular pathogenesis of human AML and build on the current base of related scientific literature. The scope of the work put forward touches on many important aspects of translational research, including an *in vivo* analysis of AML patient material, mechanistic studies using an AML cell line model system, detailed evaluation of pharmacological agents with therapeutic potential, and a high-throughput genomics approach for the identification of novel genes aberrantly silenced in this disease. In addition, these studies will provide a concept and framework for future research involving aberrant epigenetics in other hematological malignancies and solid tumors.

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